



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification⁴ : C12N 1/16, C12P 23/00 A23K 1/18 // C12R 1:645	A1	(11) International Publication Number: WO 88/ 08025 (43) International Publication Date: 20 October 1988 (20.10.88)
(21) International Application Number: PCT/DK88/00068 (22) International Filing Date: 15 April 1988 (15.04.88) (31) Priority Application Number: 1998/87 (32) Priority Date: 15 April 1987 (15.04.87) (33) Priority Country: DK (71) Applicant (for all designated States except US): DANISCO BIOTEKNOLOGI A/S [DK/DK]; Fabriksparken 58, DK-2600 Glostrup (DK). (72) Inventors; and (75) Inventors/Applicants (for US only) : FLENØ, Bent [DK/DK]; Floravej 7, DK-3660 Stenløse (DK). CHRISTENSEN, Ib [DK/DK]; Ligustervangen 7, DK-3450 Allerød (DK). LARSEN, Robert [DK/DK]; Hasselvej 32, DK-2830 Virum (DK).		(74) Agent: PLOUGMANN & VINGTOFT; Sankt Annæ Plads 11, DK-1250 Copenhagen K (DK). (81) Designated States: AT, AT (European patent), AU, BE (European patent), CH (European patent), DE, DE (European patent), DK, FI, FR (European patent), GB, GB (European patent), IT (European patent), JP, KR, LU (European patent), NL, NL (European patent), NO, SE, SE (European patent), US. Published <i>With international search report.</i>
(54) Title: ASTAXANTHIN-PRODUCING YEAST CELLS, METHODS FOR THEIR PREPARATION AND THEIR USE (57) Abstract <p>A yeast cell, preferably a mutant of <i>Phaffia rhodozyma</i>, which, when grown under conditions comprising an oxygen transfer rate of at least 30 mmoles/l/hour on Difco YM medium at 20-22°C for 5 days in 500 ml shake flasks with two baffles containing 50 ml of the medium and subjected to orbital shaking at 150 rpm, the inoculum being 100 µl of a four days old YM culture, produces the red pigment, astaxanthin, in an amount of at least 300 µg per g of yeast dry matter, typically at least 800 µg per g of yeast dry matter, determined by HPLC analysis using pure astaxanthin as a standard on a methanol extract of the yeast prepared by subjecting a suspension of 0.2 g of yeast dry matter in 20 ml of methanol to 5 x 1 minutes of disintegration at intervals of half a minute, the disintegration being performed at a temperature of at the most 20°C in a glass ball mill containing 15 g of glass balls having a diameter of 0.4 mm, the glass ball mill being provided with a cooling jacket with ice water. The amount of astaxanthin produced by the yeast cell, i.e. at least 300 and typically at least 800 µg per g of yeast dry matter, should be compared with the amounts of astaxanthin produced by known <i>Phaffia rhodozyma</i> strains which is at the most about 250 µg per g of yeast dry matter. The mutants of <i>Si(Phaffia rhodozyma)</i> may be obtained by conventional methods of mutation. The yeast cells are preferably cultivated by fed-batch fermentation which is performed under sufficient aeration, the fed-batch fermentation preferably comprising a growth phase and a subsequent growth-limited phase. Subsequent to the cultivation, the yeast cells may be subjected to e.g. rupturing and homogenization treatments, and the astaxanthin may optionally be extracted from the yeast cells. The yeast cells or the extracted astaxanthin may be used in animal feed, especially feed for anadromous fish such as salmon or trout, which when being fed with the astaxanthin-containing feed obtain an attractive red colour.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	ML	Mali
AU	Australia	GA	Gabon	MR	Mauritania
BB	Barbados	GB	United Kingdom	MW	Malawi
BE	Belgium	HU	Hungary	NL	Netherlands
BG	Bulgaria	IT	Italy	NO	Norway
BJ	Benin	JP	Japan	RO	Romania
BR	Brazil	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	LI	Liechtenstein	SN	Senegal
CH	Switzerland	LK	Sri Lanka	SU	Soviet Union
CM	Cameroon	LU	Luxembourg	TD	Chad
DE	Germany, Federal Republic of	MC	Monaco	TG	Togo
DK	Denmark	MG	Madagascar	US	United States of America
FI	Finland				

Astaxanthin-producing yeast cells, methods for their preparation and their use.

The present invention relates to astaxanthin-producing yeast strains, methods for their preparation, methods for their cultivation, and methods for isolating the astaxanthin from the yeast cells. Further,
5 the invention relates to a food or feed which contains the astaxanthin-containing yeast cells or astaxanthin recovered from these as well as a method for producing food or feed and a method for feeding animals with said feed.

It is known that the red colour of the meat of anadromous fish such
10 as salmon or sea trout is due to red pigments such as astaxanthin which is present in the feed consumed by the fish. In natural surroundings, the fish obtain their red colour from crustaceans or other astaxanthin-containing organisms, but when being bred in fish farms, the fish do not have access to these natural pigmentation sources and
15 therefore do not obtain the attractive red colour unless red pigments are supplied in the feed.

Thus, astaxanthin isolated from crustacean wastes or produced synthetically as well as other synthetic red pigments such as cantaxanthin have been used as constituents in fish feed. However, the use of can-
20 taxanthin in animal feedstuffs is prohibited in certain countries, and the synthetic astaxanthin production as well as the process for isolating natural astaxanthin are rather expensive and often also subject to seasonal variations.

Other natural astaxanthin sources are known, among these the yeast
25 *Phaffia rhodozyma* and some microalgae such as the unicellular group of green algae *Chlamydomonas nivalis* [Gert Knutson et al., "Pigmentering af laks med astaxanthin fra mikroalger", Norsk Fiskeopdræt nr. 3, pp. 4-6, 55 (1980)]. The astaxanthin produced by these organisms has been shown to confer the desired red colour to
30 anadromous fish [Eric A. Johnson et al., "*Phaffia rhodozyma* as an astaxanthin source in salmonid diets", *Aquaculture*, 20, pp. 123-134 (1980) and JP-A 57-206342]. However, the use of yeast cells in large amounts as nutrition for the fish is not desirable as this feed is

not sufficiently varied. On the other hand, the amount of astaxanthin produced by the organisms and present in a nutritionally acceptable amount of yeast cells is not sufficient to obtain the desired pigmentation, and the isolation of astaxanthin from yeast by the
5 known methods is rather expensive.

If, however, it would be possible to obtain a higher astaxanthin production from these organisms, a profitable astaxanthin production which is not subject to seasonal conditions would be possible.

The present invention provides yeast cells which contain astaxanthin
10 in sufficiently high concentrations to make it possible to use the yeast cells as or in feed for anadromous fish and other animals in which a pigmentation of the animal meat or a product of the animal is desired. The invention also provides attractive methods for obtaining astaxanthin from astaxanthin-containing yeast cells, in particular
15 the above-mentioned yeast cells having high contents of astaxanthin. Important aspects of the invention are based on a particular method for cultivating astaxanthin-producing cells and/or the provision of mutant strains with an improved inherent capability of producing astaxanthin.

20 Thus, one aspect of the invention relates to a yeast cell which, when grown under conditions comprising an oxygen transfer rate of at least 30 mmol/l/hour on Difco YM medium at 20-22°C for 5 days in 500 ml shake flasks with two baffles containing 50 ml of the medium and subjected to orbital shaking at 150 rpm, the inoculum being 100 μ l of a
25 four days old culture in YM-medium, produces astaxanthin in an amount of at least 300 μ g per g of yeast dry matter, determined by HPLC analysis using pure astaxanthin as a standard on a methanol extract of the yeast prepared by subjecting a suspension of 0.2 g of yeast dry matter in 20 ml of methanol to 5 x 1 minutes of disintegration
30 at intervals of half a minute, the disintegration being performed at a temperature of at the most 20°C in a glass ball mill containing 15 g of glass balls having a diameter of 0.4 mm, the glass ball mill being provided with a cooling jacket with ice water.

The growth conditions and determination conditions stated above are given to standardize the growth and testing methods so that the result obtained will reflect the inherent astaxanthin-producing capabilities of the yeast in question. This method has been found, by several experiments performed by the applicant company, to be a suitable and reproducible method which is easy to perform in practice. It should be noted that the determination method is not the same as the one hitherto used in the literature. The methods hitherto used in the literature, cf. e.g. Eric A. Johnson et al., "Astaxanthin formation by the yeast *Phaffia rhodozyma*", *Journal of general microbiology* 115, 1979, pp. 173-83, are based on the absorbance of a 1% (w/v) solution in acetone in a 1 cm cuvette of 1600, whereas the value, which is obtained by measuring the astaxanthin standard from Hoffmann-La Roche, is 2100. This value is based on the applicant company's own measurements as well as on the information given by Hoffmann-La Roche.

Furthermore, the known determination method measures the total pigment content of the yeast whereas the above-mentioned standard method used in the present application exclusively measures the astaxanthin content. When comparing the values obtained by the standardized method as stated above with the values stated in the literature, it should be borne in mind that the values stated in the literature will be considerably higher than the true values obtained by the standardized method stated above. Thus, whenever the present total pigment content is compared with the literature statements, a correction for the difference in extinction coefficients should be made by multiplying the total pigment content stated in the literature by 1600/2100.

The growth conditions stated above are the ones which have been found by the applicant company to be reproducible and significant for the determination of the inherent astaxanthin-producing capability. A more detailed explanation of the growth and determination conditions used for determining the inherent astaxanthin-producing capabilities of yeast strains is given in connection with the Examples.

The yeast cell according to the invention is preferably a yeast cell which belongs to the genus *Phaffia* and in particular one which belongs to the species *Phaffia rhodozyma* as this is the only *Phaffia* species known for the time being.

- 5 At present, *Phaffia rhodozyma* is the only known yeast which produces astaxanthin. The wild-type *P. rhodozyma* is isolated from deciduous tree exudates and an example of such a wild-type strain is deposited in the American Type Culture Collection under the accession number ATCC 24261.
- 10 Vegetative *P. rhodozyma* cells form buds as heterobasidiomycetous yeast. Chlamydospores are developed by budding but promycelium and proper spore formation do not occur. The chlamydospores are relatively large spherical cells with a larger lipid content than the vegetative cells. Attempts to pair the various strains in the hope of observing dikaryotic mycelium and teliospore formation have not been
- 15 successful. *P. rhodozyma* was therefore classified in the genus *Deuteromycotina* of the order *Blastomycetes* (cf. M.W. Miller et al., "Phaffia, a new yeast genus in the deuteromycotina (*Blastomycetes*)", in *International Journal of systematic bacteriology* 26:2, 1976, pp.
- 20 286-291).

- Vegetative cells are ellipsoidal (3.6-7.5) x (5.5-10.5) μm and are present in a liquid medium individually, in pairs and in some cases in short chains or small clusters. No true mycelium is developed, but a rudimentary pseudomycelium may be present. Budding occurs several
- 25 times from the same point on the cell. *P. rhodozyma* has a strong cell membrane composed by many layers, and capsule material imparts a granular appearance to the surface and causes the clustering mentioned above.

- A sexual cycle of life has not been observed. During the development
- 30 of the chlamydospores, vegetative cells are formed by budding. These cells cannot be considered to be promycelia with spores as described for *Aessosporon* (cf. J.P. van der Walt, "The perfect and imperfect states of *Sporobolomyces salminicolor*", *J. Microbiol. Serol.* 36, 1970, pp. 49-55). The chlamydospores cannot be considered to be gono-

toconter (sexually segregated spores), and their buds cannot be considered to be the haploid generation. It has not been possible by nuclear staining to demonstrate diploidization at any growth stage. Transmission electron micrographs have only shown one single nucleus during all growth phases (cf. M.W. Miller et al., op.cit.). Thus, *P. rhodozyma* is likely to be haploid, but this has not been proved.

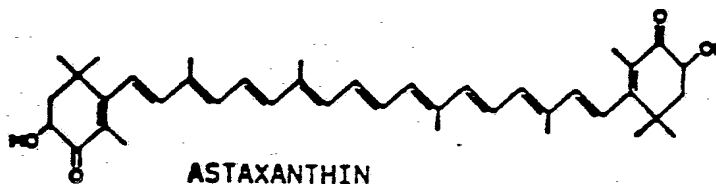
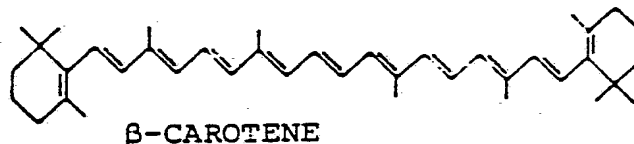
After 2-4 weeks of growth on YM agar (Difco Laboratories Incorporated, Difco manual: dehydrated culture media and reagents for microbiology, 10th Edition, Detroit 1984), the string cultures are orange to salmon-pink, depending on the strain.

P. rhodozyma has the special property of not growing at temperatures above 27°C. It ferments D-glucose, maltose, sucrose and raffinose whereas D-galactose and melibiose are not fermented. The most common carbon sources are assimilated; however, D-galactose, L-sorbose, melibiose, lactose, glycerol and citrate are not assimilated. The yeast cannot grow in vitamin-free medium without the addition of biotin (M.W. Miller et al., op.cit.). The most common nitrogen sources are assimilated, including urea. Potassium nitrate and ethylamine are not assimilated. The yeast cannot grow on 50% by weight of a glucose-yeast extract agar nor on 10% by weight of sodium chloride-yeast extract agar. The acid formation on chalk agar by the yeast is weak and so is the gelatin liquefaction. Casein hydrolysis, depolytic activity and growth in the presence of 0.1 µg of cycloheximid per ml are absent whereas the yeast is able to synthesize starch-like compounds independent of pH. The mole-% G+C is measured to be 48.3 ± 0.18 (cf. Miller et al., op. cit.).

During growth under carbohydrate- and/or nitrogen-limited conditions, when subjected to fed-batch fermentations, *P. rhodozyma* produces trehalose as a carbohydrate deposit. This is quite a new observation made by the applicant company and has not been reported hitherto.

P. rhodozyma produces a number of carotenoids, of which astaxanthin constitutes 83-87%, β -carotene 2-2.5%, echinenone 2-4% and phoenicoxanthin 5-7%, according to the literature. In practice, the ratio of astaxanthin to total pigment produced by *P. rhodozyma* has, however,

been found to vary considerably depending on the growth conditions of the yeast cells as well as the pigment determination method and has generally been found to be in the range of 50-80%.



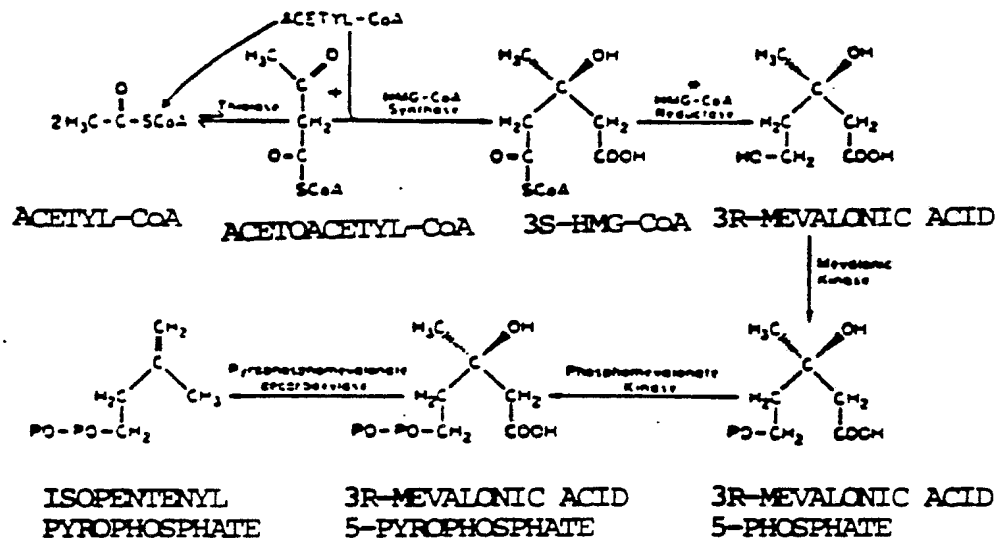
5

All hydroxylated pigments, including astaxanthin, have been described as non-bound, not as esters or other derivatives (Arthur G. Andrewes et al., "Carotenoids of *Phaffia rhodozyma*, a red-pigmented fermenting yeast", in *Phytochemistry* 15, 1976, pp. 1003-1007). There exist three optical isomeric forms of astaxanthin: (3S,3'S), (3R,3'R) and (3S,3R), each existing in various trans- and cis-configurations. It has been reported that *P. rhodozyma* only produces (3R,3'R)-astaxanthin (Arthur G. Andrewes et al., "(3R,3'R)-astaxanthin from the yeast *Phaffia rhodozyma*", op. cit., pp. 1009-1011). In the present context, "astaxanthin" is used about trans- as well as cis-configurations of astaxanthin.

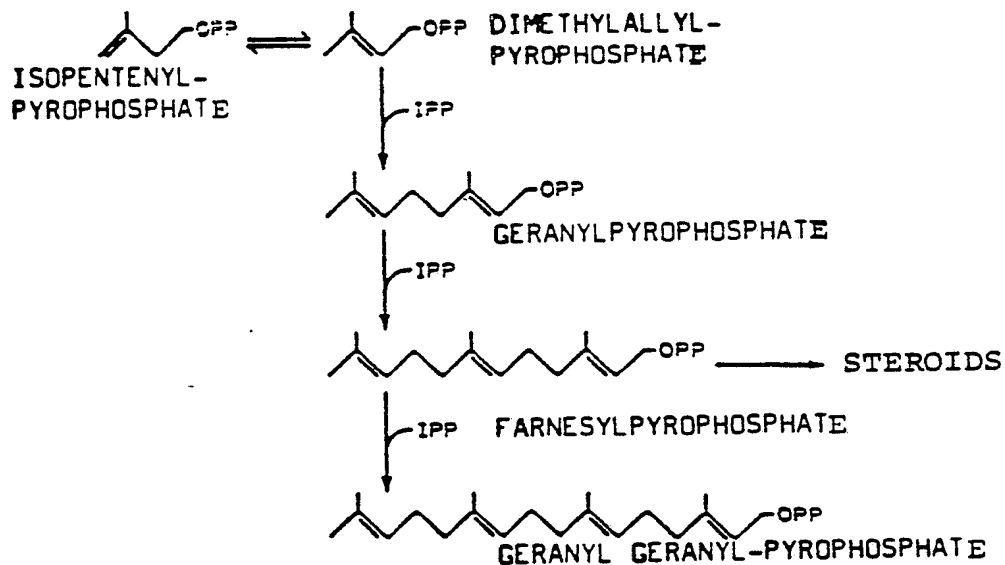
The pigment in the individual *P. rhodozyma* cells is not visible when the cells are studied in a microscope, which indicates that the pigment may be dispersed throughout the cell. However, it is also possible that the pigment is concentrated in certain parts of the cells.

Astaxanthin is an oxidated carotenoid and therefore belongs to the xanthophyl group. Similarly to other carotenoids, astaxanthin is composed of eight isoprenoid units. By the biosynthesis of astaxanthin

which is catabolite repressed, isopentenyl pyrophosphate is formed from acetyl-CoA as illustrated below.

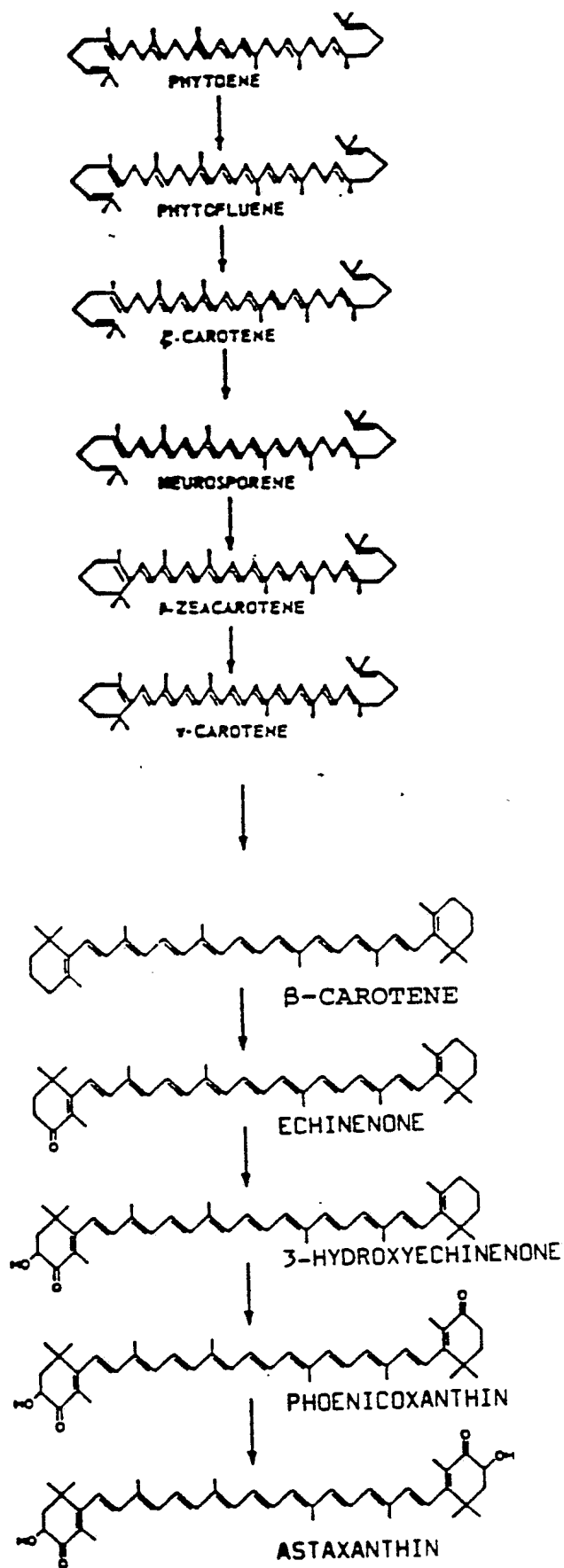


- 5 By three prenyltransferase reactions, isopentenyl pyrophosphate forms geranyl geranyl pyrophosphate via geranyl pyrophosphate and farnesyl pyrophosphate as illustrated below.



- 10 Condensation of two-molecular geranyl geranyl pyrophosphate forms phytoene which, via dehydrogenation steps and ring forming, forms astaxanthin from β -carotene. The last part of the biosynthesis has not been unambiguously determined, but Andrewes et al. (*op.cit.*) have proposed the metabolism route shown below on the basis of the pigment

composition in *P. rhodozyma*.



The enzyme system which converts geranyl geranyl pyrophosphate to astaxanthin is not known, and it is therefore not known why *P. rhodo-*
zyma produces (3R,3'R)-astaxanthin and whether there are some regu-
lating steps during this part of the biosynthesis. On the other hand,
5 the conversion of acetyl-CoA to isopentenyl pyrophosphate in other
isoprenoid-producing organisms than *P. rhodozyma* and the enzymes
which take part have been described in relatively great detail. Less
is known about the enzymes which convert isopentenyl pyrophosphate to
geranyl geranyl pyrophosphate (isopentenyl pyrophosphate isomerase
10 and prenyltransferase (J.W. Porter, S.L. Spurgeon (eds.), "Biosyn-
thesis of isoprenoid compounds". New York, 1981-1983).

The protein content of *P. rhodozyma* varies from 25 to 50% of yeast
dry matter, depending on the culturing conditions. This is a
relatively low protein content. In contrast to this, the lipid
15 content is extraordinarily high (14-27%). It is contemplated that
the nucleic acid constitutes 8% similarly to other yeasts and that
the amino acid composition is similar to the composition in other
known yeasts such as *Saccharomyces cerevisiae* and thus has a very low
content of certain amino acids, e.g. methionin and cystein (cf.
20 Gerald Reed and Henry J. Peppler, *Yeast Technology*, 1973, p. 329,
published by The AVI Publishing Company, Inc.). This and the overall
yeast composition which comprises a high amount of nucleic acids make
the yeast inconvenient for animal nutrition purposes when the yeast
is the only nutrient source, such as indicated above. Thus, without
25 addition of certain amino acids and other nutrient components, the
yeast will not be a suitable major nutrition component for fish or
other animals.

The total amount of astaxanthin which is produced by the wild type *P.*
rhodozyma when this is grown under the normal known conditions is
30 sufficient to confer a red colour to the yeast cell but is not suffi-
cient to make recovery of the astaxanthin from the yeast cells econo-
mically feasible.

None of the *Phaffia rhodozyma* species described in the literature have an inherent astaxanthin-producing capability of more than 300 μg per g of yeast dry matter when analyzed in accordance with the above standard methods, vide Table 2 and Table 6 of the examples. However, according to the present invention, it has been found possible to obtain yeast cells which are inherently capable of producing astaxanthin in an amount of at least 450 μg per g of yeast dry matter, such as at least 600 μg per g of yeast dry matter, preferably at least 700 μg per g of yeast dry matter, more preferably at least 1000 μg per g of yeast dry matter, especially at least 1500 μg per g of yeast dry matter, and most preferably at least 2000 μg per g of yeast dry matter, the growth and the determination being performed by the standard methods stated above. These yeast cells have been produced from naturally occurring *Phaffia rhodozyma* by mutagenization. Thus, an aspect of the present invention relates to a method for producing a yeast cell showing the high inherent astaxanthin-producing capability explained above, the method comprising treating a yeast cell with a mutagen and selecting a resulting mutant which, when grown under the conditions stated above, is capable of producing astaxanthin in an amount of at least 300 μg per g of yeast dry matter, determined by the method stated above.

The mutagenization may be performed as a single mutagenization, but it has been found advantageous to perform two or more consecutive mutagenizations, as it has been found that the inherent capability of producing astaxanthin may be improved by each mutagenization step. The starting yeast cell subjected to mutagenization is normally a yeast cell which, when grown under the conditions stated above, produces astaxanthin in an amount of less than 300 μg per g of yeast dry matter, determined by the method stated above, but it is evident that a normal candidate for the mutagenization treatment will be a naturally occurring yeast cell having as high inherent astaxanthin production as possible. Such yeast cells are normally yeast cells which belong to the genus *Phaffia*, in particular yeast cells belonging to the species *Phaffia rhodozyma*, such as is mentioned above.

The mutagenization treatment may be performed using any suitable mutagen (in the present context, the term "mutagen" is to be understood

in its broad sense as comprising, e.g., not only agents which have a mutagen effect, but also treatment which have a mutagen effect such as UV irradiation). Examples of suitable mutagens are ethyl methane sulphonate, UV irradiation, N-methyl-N'-nitro-N-nitrosoguanidine, nucleotide base analogues such as bromouracil, and acridines, but it is contemplated that any other effective mutagen will be suitable for the treatment.

In accordance with conventional mutagenization techniques, the mutagenization is followed by suitable selection of the cells which have the highest astaxanthin production. Due to the fact that astaxanthin is a pigment, this selection may be performed relatively easily by normal visual means, such as simple observation of single colonies. An alternative method is to perform analysis on cultures made from single colonies, e.g. by using the standardized cultivation conditions and determination conditions as explained above.

Two strains produced by the mutagenization method according to the invention and showing a particularly high astaxanthin productivity were deposited on 6 April, 1987 at the Centraalbureau voor Schimmelcultures, Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, the Netherlands (CBS) under the accession Nos. 224-87 and 225-87, respectively, and one strain being a reisolat of CBS 225-87 (vide Example 1) was deposited on 23 March, 1988 at CBS under the accession No. 215-88, and an aspect of the invention relates to these yeast strains as well as mutants or derivatives thereof which have substantially retained or improved the astaxanthin-producing capability of these strains.

The invention also relates to a method for producing astaxanthin-containing yeast cells or cell parts, or astaxanthin derived from these yeast cell or cell parts. This method comprises cultivating astaxanthin-producing yeast cells under aerobic conditions in a medium containing carbohydrate sources, assimilable sources of nitrogen and phosphorus, micronutrients and biotin or desthiobiotin at a temperature in the range of 15-26°C so as to obtain a biomass containing astaxanthin in an amount of at least 300 µg per g of yeast dry matter, determined by the method stated above,

and optionally performing one or several of the following steps in arbitrary sequence:

- harvesting cells from the culture so as to obtain a yeast cream,
- opening the cells, for example rupturing the cell walls by means of mechanical, chemical and/or enzymatic treatment and/or
5 subjecting the cells to sonication, autolysis, osmolysis and/or plasmolysis optionally with addition of suitable agents such as detergents, acids, bases, enzymes, autolysis-enhancing substances, osmolysing agents such as salts, and/or plasmolysing agents,
10
- homogenizing the cells to obtain a homogenate,
- drying the cells, the cell fragments or the homogenate, preferably to a water content of at the most 12% by weight, preferably at the most 10% by weight,
- 15 - extracting astaxanthin from the cells, the cell fragments or the homogenate.

The amount of astaxanthin stated above, 300 μg per g of yeast dry matter, is higher than any astaxanthin concentration reported in the literature. Although Johnson et al., *op. cit.*, reports an astaxanthin
20 content of 295 μg per g of yeast dry matter, this value does not only comprise the astaxanthin content but in fact the total pigment content of the yeast cell. Further, this pigment content was measured using a value of the absorbance of a 1% (w/v) solution in acetone in 1 cm cuvette of 1600 which is lower than the one measured by the
25 present applicants (2100) so that the value reported by Johnson et al. corresponds to at the very most $295 \times 1600 / 2100 = 225 \mu\text{g}$ of total pigment (not only astaxanthin) per g of yeast dry matter. This pigment content from the literature should be compared with the total pigment content of the yeast strains of the present invention which
30 is 885 $\mu\text{g/g}$ of yeast dry matter for the strain CBS 224-87, 1176 $\mu\text{g/g}$ of yeast dry matter for the strain CBS 225-87, and about 1340 $\mu\text{g/g}$ of yeast dry matter for the strain CBS 215-88, or even higher, e.g. at least 2000 $\mu\text{g/g}$ of yeast dry matter.

The high astaxanthin concentration in the yeast cells of the
35 invention may be obtained partly by the use of special cultivation

conditions as explained below and partly by selecting a yeast strain with a high inherent astaxanthin productivity, preferably a yeast strain as discussed above, and in particular it is preferred to combine the special cultivation conditions and the use of special
5 astaxanthin-producing yeast strains.

The cultivation is preferably performed as a fed-batch fermentation under conditions where substantially no alcohol is formed. As mentioned above, the temperature of the culture is in the range of 15-26°C. Below 15°C, the growth tends to be too slow to be acceptable
10 for industrial production, and above 26°C, the viability of the culture is severely impaired. The preferred temperature range is 20-22°C.

The fermentation or at least part thereof is normally performed in a medium which comprises suitable macro- and micronutrients for the
15 cells, such as molasses or saccharose as a carbohydrate source and nitrogen sources such as corn-steep-liquor, diammonium sulphate, ammonium phosphate, ammonium hydroxide or urea, phosphorus sources such as ammonium phosphate and phosphoric acid and added micro-nutrients or mineral salts such as magnesium sulphate, zinc sulphate
20 and biotin or desthiobiotin. The molasses or saccharose are preferably supplied to the medium separately from the other components in accordance with conventional methods used in yeast production. When the medium comprises molasses, it has been found that the growth of the yeast cells is affected by the concentration of sugar or other
25 growth-inhibiting substances therein in the fermenter. This effect has not been observed when the medium comprises corn-steep-liquor or solids. Accordingly, it may prove advantageous to regulate the fermentation so that the concentration of sugar (expressed as the total concentration of glucose and saccharose) in the fermenter is at
30 the most 8 g/l, preferably at the most 5 g/l, and most preferably at the most 1 g/l.

The culture is aerated during the total fermentation, i.e. it is grown under aerobic conditions. By the term "aerobic conditions" is meant that the oxygen supply should be sufficient so that substan-
35 tially no oxygen limitation will occur during the fermentation.

According to a special aspect of the invention as indicated above, the concentration of astaxanthin in the biomass obtained is increased by performing the cultivation under selected conditions. These conditions involve a cultivation which comprises a growth phase under conditions which are substantially sufficient with respect to substantially all growth conditions and a subsequent growth-limited phase. The growth-limited phase is preferably established by providing conditions where the growth medium under continued aeration is deprived of at least one growth factor so as to enhance the production of astaxanthin during the subsequent phase.

The growth-limited phase should be understood to generally mean the phase in which the main part of the cells have stopped growing. This phase does of course occur when the medium is deprived of at least one growth factor but is also observed during the last part of the period of carbohydrate addition when the amount of cells present in the fermenter is well in excess of the aeration capacity of the fermenter.

It is not known why the subsequent growth-limited phase has the surprising effect of considerably enhancing the production of astaxanthin (for example from 231 to 369 μg per g of yeast dry matter as obtained in one of the examples which follows), but it is contemplated that the precursors of astaxanthin have been produced during the growth phase, and that the subsequent growth-limited phase provides conditions which promote the final production of astaxanthin, possibly oxidizing conditions with the surplus of oxygen which becomes available when the growth is terminated. At any rate, it seems essential that aeration is continued during the subsequent growth-limited phase. The duration of the subsequent growth-limited phase is preferably at least about 16 hours, such as 16-24 hours, as shorter durations may tend to decrease the extra effect obtainable, whereas there seems to be no substantial effect obtainable by extending the growth-limited phase to more than about 24 hours.

Expressed in a functional manner, the conditions of the growth-limited phase should be adapted to enhance the astaxanthin production to

at least 1.2 times the production obtained without the subsequent phase, such as at least 1.3 times the production obtained without the subsequent phase, preferably at least 1.4 times the production obtained without the subsequent phase and most preferably at least 1.5 times the production obtained without the subsequent phase.

While the yeast cell subjected to the special cultivation with the subsequent growth-limited phase may be a wild-type astaxanthin-producing yeast cell whose astaxanthin productivity is increased due to the subsequent growth-limiting step, such as a wild-type yeast cell of the genus *Phaffia*, in particular of the species *Phaffia rhodozyma*, it is preferred that the yeast cell subjected to the cultivation is a yeast cell having an inherent and improved capability of producing astaxanthin, typically a yeast cell obtained by mutagenization as explained above. With these yeast cells with an inherent increased astaxanthin production, the concentration of astaxanthin in the biomass obtained when using the special cultivation method comprising a growth-limited phase may be at least 600, preferably at least 800, more preferably at least 1000 μg per g of yeast dry matter, especially at least 1500 μg per g of yeast dry matter, e.g. at least 2000 μg per g of yeast dry matter, and most preferably at least 3000 μg per g of yeast dry matter, determined as stated above.

Normally, and as used above, the total pigment content and astaxanthin content of the yeast cells or yeast cell parts are stated as $\mu\text{g/g}$ of yeast dry matter. However, other ways of stating the total pigment and astaxanthin content may be found convenient. It may, e.g., be useful to state the total pigment content and astaxanthin content as $\mu\text{g/ml}$ of the suspension in which it is present, e.g. in the growth media. Thereby, it will not be necessary to determine the weight of yeast dry matter of the yeast cells from which the astaxanthin or total pigment is recovered. Thus, during the fermentation or cultivation of the yeast cells, the astaxanthin and/or total pigment content of the yeast cells may easily be determined.

After the cultivation as described above to obtain yeast cells having a high astaxanthin content, the culture may be subjected to the sub-

sequent treatments mentioned above to isolate the yeast cells and/or condition them for their subsequent use, such as by rupturing the cells, and/or astaxanthin may be extracted from the cells.

5 The following important examples of these treatments are discussed in greater detail:

The cells may be ruptured by subjecting the cells to an increased pressure and then releasing the pressure.

10 The cells may be subjected to the increased pressure and release of the pressure by passage through a system comprising a valve homogenizer where the increased pressure is built up in front of the valve homogenizer. The valve homogenizer typically comprises an aerojet through which the cell suspension is passed under high pressure and an obstruction member which the jet hits substantially after passage through the valve. Examples of cell disruption valves are described
15 in APV Gaulin Technical Bulletin No. 74 of March 1985 (APV Gaulin International SA, P.O. Box 58, 1200 AB Hilversum, the Netherlands), incorporated by reference herein. As an example of a suitable cell rupture homogenizer may be mentioned an APV Gaulin MC4 homogenizer with a cell rupture valve of the type CR as described in the
20 above-mentioned publication. The homogenizer is connected to a heat exchanger in which the suspension comprising ruptured cells passes from the cell rupture valve. The pressure of the cell suspension in front of the valve may, e.g., be about 400-1200 bar, such as, e.g., about 700 bar. This treatment may for example be repeated three times
25 with intervening cooling of the homogenate in the heat exchanger.

As is explained below, it is necessary that the cells are ruptured or otherwise treated when they are to be used in feed as the utilization of the astaxanthin content to a high degree depends on the cell contents being available to the digestive system of the
30 animal in question. Thus, substantially no pigmenting effect is obtained when feeding fish with feed containing non-ruptured astaxanthin-containing cells.

The ruptured yeast cells may be subjected to ultrafiltration or evaporation so as to concentrate the ruptured cells. The ultrafiltration may, e.g., be performed in a lab unit system available from De Danske Sukkerfabrikker, for example a System 37 which comprises three filtering units of a total filter area of 0.88 m² of an ultrafiltration membrane of the type RC 70. Another method for concentrating the ruptured cells is to perform vacuum evaporation of water from the cell suspension.

The ruptured cells may be dried by spray drying or drum drying. Before drying, carriers such as sodium caseinate, antioxidants and/or emulsifiers are preferably added. Spray drying may, e.g., be performed by subjecting a homogeneously mixed slurry of the ruptured cells and optionally a carrier such as sodium caseinate, preferably in the form of an aqueous solution, to spray drying. The spray drying may suitably be carried out by mixing the aqueous sodium caseinate solution with the yeast slurry so as to obtain a sodium caseinate concentration of about 2-10% (w/v). The resulting mixture is then allowed to stand with stirring in a nitrogen atmosphere before being pumped into a spray drying tower in which it is subjected to drying at a temperature of, e.g., 150-230°C, such as about 180°C to decrease the water content of the yeast cell material to e.g. at the most 10% by weight. The yeast cell material is then subsequently atomized by means of a spray wheel. The powdery yeast material resulting from the spray drying treatment is suitably recovered by means of cyclone and optionally subsequently sieved and packed. An example of a suitable spray drying equipment is a spray tower of the type EAK-1 from Anhydro. As an alternative, the ruptured yeast cells may be subjected to drum drying, for example in a closed drum drying equipment at a temperature of 150-200°C.

As the astaxanthin is very easily decomposed at high temperatures, it is important that the ruptured yeast cells are subjected to high temperatures for as short a time as possible. Further, as astaxanthin is sensitive to oxygen, the drying should preferably be performed under non-oxidizing conditions, for example in an inert atmosphere such as water vapour (which may be the water vapour evaporated from the yeast suspension), nitrogen, and/or carbon dioxide.

Prior to drying, the ruptured cells are optionally mixed with suitable emulsifiers such as sorbitan monostearate or antioxidants, butyl hydroxytoluene (BHT), butyl hydroxyanisol (BHA), vitamin E, ascorbic acid, (II) sulphate or (II) phosphate esters of ascorbic acid, or
5 ascorbyl palmitate.

Dried ruptured cells are immediately useful as a constituent of animal feed, such as is explained below.

The astaxanthin content of the yeast cell may be extracted from these by use of various extraction agents and extraction procedures - so as
10 to ensure that a substantial total extraction of the astaxanthin from the yeast cells is obtained. In most cases, the extraction has to be performed in ruptured cell material. Thus, the ruptured cell material, which may be dry or wet, may be extracted with an organic solvent such as petroleum ether which is suitably employed in the
15 case of wet cell material as the petroleum ether forms a phase separately from the water phase. Other suitable organic solvents are acetone or alcohols such as methanol or ethanol, ethers, ketones and chlorinated hydrocarbons. By the extraction, astaxanthin is dissolved in the organic solvent. The astaxanthin may be obtained by removing
20 the solvent from the solution such as by evaporation in a falling film evaporation system before drying. However, also a concentrate of astaxanthin in the organic solvent may be convenient for certain purposes. A concentrate may be used per se in the production of feed or food, or the concentrate may be diluted and used in the diluted
25 state in the preparation of feed or food, for example by impregnating feed or food constituents with the solution or by using the solution (or the concentrate) for colouring food constituents such as oils or fats.

The astaxanthin may also be extracted from yeast cells by use of
30 carbondioxides under supercritical conditions. The carbondioxide may optionally be used in combination with suitable entrainers such as organic solvents, especially solvents of the above mentioned types, or solvents such as chloroform or acetonitrile, or glacial acetic acid. The yeast cells subjected to supercritical extraction may be

wet or dry whole yeast cells or ruptured, e.g. homogenized, yeast cells.

A preferred method of isolating whole astaxanthin-containing cells from the culture is to filtrate the yeast cream, for example on a filter press or a rotating drum filter, so as to obtain a filter cake, e.g. with a dry matter content of about 25-35%. The filter cake may then suitably be extruded into strings, for example strings with a diameter of about 0.5-2.0 mm in an extruder equipped with a perforated plate, so as to obtain strings consisting of yeast particles. The strings are preferably extruded directly into the hot air in a fluid bed where they are dried. The evaporation in the fluid bed is preferably regulated so that the temperature of the yeast particles is kept below 50°C such as at 30-40°C, and the process is terminated when the water content is brought down below 10% by weight, preferably below 8%, as determined by the yeast dry matter content (the procedure is described in the Examples). Alternatively, the drying may be performed in a tray drier under the same conditions as in the fluid bed. The dried whole cell material may then be comminuted in a ball stirring mill such as a Coball® mill after which it is subjected to extraction.

According to a special method, whole cell dried material, for example obtained as described above, may be mixed with an oily phase such as an edible oil or fat such as soy bean oil or fish oil, or another organic solvent such as a solvent of the type discussed above. The temperature is preferably in the range of 20-30°C. The mixture obtained from the cell material and the oily phase or the organic solvent may be ground in a mill such as a ball mill, e.g. a ball stirring mill such as a Coball® mill, to rupture the cells and release astaxanthin from the cells. The resulting suspension may be used as such in feed, or the oily phase containing the astaxanthin may be separated from cell residues before use. The separation is suitably performed by centrifugation in a fast running centrifuge, the same principle which is employed in separation of bacteria from wort. Another possibility is of course to mix ruptured dried cell material obtained by the methods discussed above with an oily phase in a similar manner to extract the astaxanthin into the oily phase

and perform separation as described above. The oily phase may be used for colouring feed in the same manner as described above.

In contrast to most conventional extraction procedures which, as stated above, has to be performed on ruptured cell material, it has been found that glacial acetic acid successfully may be employed to extract astaxanthin from whole, non-ruptured yeast cells. Thus, according to one aspect of the present invention, astaxanthin may be extracted from whole yeast cells with a solvent comprising glacial acetic acid, the extraction preferably being performed at a temperature above the freezing point of the solvent, e.g. in the range of 20-100°C, preferably in the range of 20-80°C, and more preferably in the range of 20-60°C. It is contemplated that it is possible to obtain a more selective extraction of astaxanthin when the extraction is performed at the lower temperatures as concomitant extraction of fat and other extractable components will be limited at these low temperatures. The concentration of glacial acetic acid in the solvent is preferably in the range of 5-100, 10-70. The extraction with glacial acetic acid results in an extraction of the pigment of the cells of about 70-90%, i.e. substantially all the pigment and astaxanthin contents of the yeast cells are found in the glacial acetic acid extract. In addition, the extract normally contains about 30-35% of yeast dry matter. Suitably, the yeast subjected to extraction with glacial acetic acid is in the form of dried yeast, e.g. yeast which has been filtered and subsequently extruded into a fluid bed wherein it is dried, as thus treated yeast cells will not rupture during the extraction treatment (unless the extraction treatment involves vigorous mechanical treatment of the yeast cells). This will facilitate the subsequent separation of the extract containing the pigment from the yeast cells as compared with extraction of ruptured or homogenized cells, which, due to their relatively small sizes in comparison with non-ruptured cells to a large extent tend to block up the pores of the filter employed. The glacial acetic acid extraction is illustrated in Example 9. Extraction of wet yeast cells with glacial acetic acid may also prove useful.

- The extracted astaxanthin as well as the whole dried cell material are preferably kept under oxygen-deficient conditions so as to protect the astaxanthin from decomposition. Thus, the astaxanthin-containing yeast cells or the extracted astaxanthin is preferably protected by means of antioxidants such as butyl hydroxyanisol (BHA), butyl hydroxytoluene (BHT), vitamin E or ascorbic acid, (II) sulphate or (II) phosphate esters of ascorbic acid, or ascorbyl palmitate, and/or emulsifiers such as monoglycerides or sorbitan esters and are suitably kept under hermetic conditions.
- 10 The invention also relates to an animal feed comprising yeast cells or yeast cell parts containing astaxanthin in an amount of at least 300 μg per g of yeast dry matter, determined as explained above, in combination with other feed constituents. Preferably, the astaxanthin-containing yeast cells or yeast cell parts constitute at the most 10% by weight of the dry matter of the total animal feed composition, preferably at the most 5% and more preferably at the most 3%. These values are calculated on the final feed to be administered to the animals. It is also possible to prepare feed premixes having a higher concentration of yeast cells. The yeast cells or yeast cell parts or the astaxanthin is optionally admixed with emulsifiers which are capable of making the astaxanthin dispersible in water. In addition, the astaxanthin-containing yeast cells or yeast cell parts may be protected against oxidation by means of the antioxidants and/or emulsifiers mentioned above, and/or the animal feed may be packaged in air-tight and optionally evacuated containers.

The astaxanthin-containing dried yeast cells may also be packaged per se for use as a feed constituent, the final feed mixture being prepared at the site of use, or the yeast cells being administered per se to animals which are otherwise fed with normal or adapted feed mixtures.

The yeast cells or yeast cell parts are suitably and normally mixed with other nutrient components which are preferably selected from protein and carbohydrate sources, fats or oils and micronutrients such as vitamins and minerals. As examples of protein sources may be

mentioned casein, albumin, wheat gluten, fish meals, concentrated fish residues (fish glue meal and blood meal). As examples of carbohydrate sources may be mentioned gelatinized starch, extruded wheat, molasses, vegetable flours and corn starch. The fat constituents in the feed may for example be fish oil and cod liver oil and/or vegetable oils such as corn oil. The minerals may be selected, e.g., from inorganic or simple organic compounds of calcium, phosphorus, sodium, potassium, chlorine, magnesium, copper, manganese, zinc, cobalt and selenium. As examples of vitamins may be mentioned vitamin B₁₂, proline, vitamin A, vitamin D, vitamin E, vitamin K, thiamine, ascorbic acid, riboflavin, pyridoxine, pantothenic acid, niacin, biotin, choline and inositol.

The invention also relates to food or feed comprising astaxanthin which has been extracted from yeast cells, for example by any of the methods described above, preferably from yeast cells according to the invention or yeast cells produced by the method of the invention. The astaxanthin may be used in admixture with the feed constituents described above and also in admixture with other food or nutrient components as well as in admixture with other colourants. Thus, astaxanthin extracted from yeast cells is well suited alone or in combination with other colourants for use in edible oils, butter, margarine, shortening, mayonnaise, patés, soups, snack products, surimi-based products, desserts, ice cream, confectionery, baked products, and beverages. When the astaxanthin is used in food which is mostly constituted by water or water phases, the astaxanthin is preferably mixed with an emulsifier as discussed above which makes the astaxanthin dispersible in the water phase without any tendency to crystallize and without the necessity of adding an oily phase to dissolve the astaxanthin.

Furthermore, the invention relates to a method for feeding animals to obtain a reddish pigmentation of their meat and/or of products produced by the animals, comprising administering to the animals a feed containing yeast cells or cell parts containing astaxanthin in an amount of at least 300 µg per g of yeast dry matter, determined by the method stated above, or astaxanthin extracted from such yeast cells or cell parts.

The amount of the feed containing the astaxanthin or the astaxanthin-containing yeast cells or cell parts administered to the animals will depend upon the animal species in question and upon the pigmentation effect which it is desired to obtain by means of the astaxanthin. Evidently, the principle to be followed is that the animal should have a normal recommended daily ration of macro- and micronutrients and, in addition, astaxanthin in a form and an amount which will result in the desired pigmentation of the animal meat or the animal product in question. In some cases, the amount of astaxanthin to be administered will depend on the season; thus, for example, it will normally not be preferred to administer astaxanthin or other carotenoids to cows to obtain a pigmentation of the butter in the summertime as the butter pigmentation is normally considered adequate when the cows are grazing. Also the amount in which the feed containing the astaxanthin or the astaxanthin-containing yeast cells or cell parts is administered to the animals may in some cases be dependent on the season. Thus, for example in the case of fish such as salmon or sea trout, the amount of feed consumed by the fish in the wintertime is relatively low which is in contrast to the amount consumed by the fish in the summertime. However, a suitable amount of feed administered to the fish may be about 1.5% of fish body weight per day which corresponds to the recommendations given by the California State Department of Fish and Game.

When feeding poultry by the method stated above in order to pigment the yolks of the eggs produced by the poultry and/or the meat or skin of the poultry, the feed may be constituted by conventional poultry feed components, an example of which is one which is preferably constituted by protein and carbohydrate sources such as soy bean meal, soy bean protein, cellulose, starch and fat sources such as soy bean oil, vitamins such as an overall vitamin mix and minerals such as a mixture of the common mineral components for poultry as well as calcium sources for the egg shells, the calcium sources preferably being calcium carbonate and calcium hydrogen phosphate. A small amount of sodium chloride may also be present. The feed may be administered in a conventional dosage.

The invention is further illustrated in the following Examples:

MATERIALS AND METHODS

Maintenance of cultures

Cultures of *Phaffia rhodozyma* are maintained in two ways:

- 5 1) On agar slants (YM-agar). The slants are incubated for one week at 20°C and maintained at 4°C for 1 month, recultivated in YM-broth, before new slants are made.
- 10 2) Cryopreservation at -80°C. From cryovials or agar slants the strains are inoculated in 50 ml of YM-broth in 250 ml shake flask. The shake flask is incubated on an orbitshaker (150 rpm) at 20°C for 4-5 days. The yeast cells are allowed to settle and the liquid is decanted. The sediment is mixed with glycerol to a concentration of 20%, dispensed in cryovials and stored in a deep freezer at -80°C.

15 *Determination of yeast dry matter content*

5 ml of a yeast cell culture are centrifuged in a weighed out Sarstedt tube (which has been dried to constant weight at 110°C) at 10,000 x g for 5 minutes and washed twice in demineralized water. The liquid is removed by decantation and the weight of the tube with the
20 cells is measured after drying to constant weight at 110°C, which gives the weight of the yeast cells (Y g). The yeast dry matter content (YDMC) is then calculated as:

$$\text{YDMC (g/l)} = Y/5.00 \times 1,000$$

The content stated being the mean value of two determinations.

25 *Spectrophotometrical analysis for total pigment determination*

The total pigment content in a methanol extract is determined spectrophotometrically by means of λ_{\max} and Beer's law as described by B.H. Davies, "Carotenoids", in T.W. Goodwin (ed.), *Chemistry and Biochemistry of plant pigments*, New York, 1976, Vol. 2, p. 149. The spectrophotometer employed is a Shimadzu UV visible recording spectrophotometer UV 260. Pigment content is calculated by using formulas 1, 1a, 2, 2a below and the extinction coefficients of Table 1 below.

TABLE 1

Extinction coefficients of the astaxanthin standard in different solvents prepared as stated for the standard solution above

Solvent	Absorption maximum	E _{1%} _{1cm}
Acetone	475	2105
Methanol	472	2100
Ethanol	476	2100
Glacial acetic acid	482	1856

E_{1%}_{1cm} = absorbance of 1% (w/v) solution in a 1 cm cuvette.

Pigment extraction and analysis - Method 1

About 30 ml of the yeast culture were transferred to Sarstedts tubes and centrifuged for 5 minutes at 10,000 x g. The yeast cells were washed in demineralized water and suspended in about 20 ml of methanol. To a glass ball mill of the type Bead Beater (Biospec Products Inc., USA) in which the rotor was covered with glass balls with a diameter of 0.4 mm (about 15 g of glass balls), the methanol suspension was added so as to occupy the remaining free ball mill volume. Disintegration treatment was carried out by running the mill 5 times for 1 minute at intervals of 30 seconds, ice water being kept in the cooling jacket so as to ensure that the temperature of the disintegration treatment was kept below 20°C. Immediately after the disintegration treatment, a part of the homogenate was transferred

to Sarstedt tubes, and the yeast dry matter was determined as described above, but without centrifugation. A known amount (b g) of the homogenate was transferred to a 10 ml measuring flask and solvent was added to give 10 ml. Absorbance was measured in this solution.

- 5 The total pigment content in μg per g of yeast dry matter is determined by:

$$X = \frac{E \times 10 \text{ ml}}{\frac{1\%}{E_{1\text{cm}}} \times b \times D} \times 1,000,000 \quad (1)$$

10

E = absorption at λ_{max} in the solvent used in a 1 cm cuvette

$\frac{1\%}{E_{1\text{cm}}}$ = absorbance of 1% (w/v) solution in a 1 cm cuvette

b = g of extract

- 15 D = mg of yeast dry matter/g extract

Pigment extraction and analysis - Method 2

- A predetermined amount of the yeast culture (a ml) were transferred to Sarstedts tubes and centrifuged for 5 minutes at 10,000 x g. The yeast cells were washed in demineralized water and suspended in about
- 20 20 ml of methanol. To a glass ball mill of the type Bead Beater (Biospec Products Inc., USA) in which the rotor was covered with glass balls with a diameter of 0.4 mm (about 15 g of glass balls), the methanol suspension was added so as to occupy the remaining free ball mill volume. Disintegration treatment was carried out by running
- 25 the mill 5 times for 1 minute at intervals of 30 seconds, ice water being kept in the cooling jacket so as to ensure that the temperature of the disintegration treatment was kept below 20°C. Immediately after the disintegration treatment, the liquid was transferred to a 50 ml measuring flask. The glass beads are washed in the mill with 4
- 30 x 8 ml of methanol. The fractions are collected and mixed and

methanol is added to give 50 ml. The methanol extract is filtered before pigment analysis. The yeast dry matter in the culture is determined as described above.

The total pigment content pr. ml sample is determined by:

$$5 \quad X' = \frac{E \times 50 \text{ ml}}{\frac{1\%}{E_{1\text{cm}}} \times a} \times 10.000 \quad (2)$$

X' = μg of pigment/ml of in sample

10 a = volume of sample in ml

E = absorbtion at λ_{max} of the solvent used in a 1 cm cuvette

$\frac{1\%}{E_{1\text{cm}}}$ = absorbance of 1% (w/v) solution in a 1 cm cuvette

The total pigment content per g of yeast dry matter is determined by

$$15 \quad Y = \frac{X'}{\text{YDMC}} \times 1.000 \quad (2a)$$

Y = μg of pigment/g yeast dry matter

YDMC = g yeast dry matter/l culture broth

20 *HPLC analysis for astaxanthin determination - Method 1*

HPLC data:

Equipment:

Columns: LKB Ultropac Precolumn, Lichrosorb RP 18 7 μm ,
4x30 mm.

25 LKB Ultropac Column, Lichrosorb RP 18 5 μm , 4x250
mm.

Detector: LKB 2151 variable wavelength monitor.

Integrator: Waters 740 Data Module.

Controller: LKB 2152 HPLC Controller.

Pumps: LKB 2150 HPLC Pumps.

Autosampler: LKB 2157 autosampler with variable loop.

Manual inj.: Rheodyne 20 μ l loop.

5 **Solvents:** A: 860 ml of acetonitril + 100 ml of water + 40 ml of formic acid.

B : 960 ml of ethylacetate + 40 ml of formic acid.

All solvents were of HPLC quality.

10 *Flow:* 1.0 ml/min.

Gradients: 0-100% B 20 minutes, linear gradient.
 100-0% B 10 minutes, linear gradient.

Detector: 471 nm.

Temperature: Ambient temperature.

15 *Standard solution:* 5 mg of pure astaxanthin (mp 220-222°C, absorbance of 1% v/w acetone solution in a 1 cm cuvette 2100) supplied by Hoffmann-La Roche (hereinafter referred to as the astaxanthin standard) are weighed out and dissolved in 500 ml of acetone.

For the HPLC analysis, 20 μ l of the sample in question is injected
20 into the HPLC chromatograph.

HPLC analysis for astaxanthin determination - Method 2

HPLC data:

Equipment:

5 Columns: Supelco precolumn
Supelco LC 18 - DB, particle size 5 μ
Column dimensions 4.6 x 250 cm

Detector: LKB 2151 variable wavelength monitor
Integrator: Waters 740 Data Module
Controller: LKB 2152 HPLC Controller
10 Pumps: LKB 2150 HPLC pumps
Autosampler: LKB 2157 autosampler with variable loop
Manual inj.: Rheodyne 20 μ l loop

Solvents: A: 400 ml tetrahydrofurane
400 ml methanol
15 200 ml 0.02 M glycinebuffer pH = 2.6
B: 1000 ml tetrahydrofurane

All solvents except the buffer are of HPLC grade. The buffer is sterilfiltered through a 0.22 μ m filter before use.

20 Detector: 480 nm
Temperature: Room temperature

Gradients and flow:

	Solvent	%	Time min.	Flow ml/min.
5	B	0	0-11	1.0
	B	0-90	11-21	1.0
	B	90	21-29	1.5
	B	90-50	29-31	1.5
	B	50	31-32	1.0
10	B	50-0	32-35	1.0
	B	0	35-39	1.0

Standard solution: 5 mg of astaxanthin standard are weighed out and dissolved in 500 ml tetrahydrofuran.

- 15 For the HPLC analysis, 20 μ l of the sample in question is injected into the HPLC chromatograph.

Sarstedt tubes

Polypropylene centrifuge tubes provided with a polypropylene stopper of the type 55533 supplied by Hounisens Laboratory, Århus, Denmark.

20 *Chemicals*

Chemicals used in a laboratory scale were of analytical grade.
Chemicals used in fermentations were of food grade.

The glucose and saccharose concentrations were analyzed by use of kits (Best. No. 139041) from Boehringer Mannheim.

25 *Medium for shake flask cultivations and agar plates*

YM(Yeast Morphology) medium supplied by Difco Laboratories Incorporated (Difco Manual: Dehydrated culture media and reagents for microbiology, 10th edition, Detroit, 1984).

Cryo vials

Polypropylene tubes with a volume of 2.0 ml of the type 363401 supplied by Technunc, Roskilde, Denmark.

EXAMPLE 1

5 *Mutagenization*

In each case, the mutagenization treatment was carried out so as to obtain a degree of survival of 1-5% of the treated culture. Suitable mutants were selected by visually comparing the intensity of the red colour of the mutants when plated as single colonies on the agar
10 plates.

UV-irradiation

A just turbid four days old culture of ATCC 24261 grown in YM medium at 20-22°C was diluted in a 0.9% NaCl solution to concentrations of 10^{-1} , $10^{-1.5}$, 10^{-2} , $10^{-2.5}$ and 10^{-3} , respectively, and 0.3 ml of each
15 of these dilutions was plated on agar plates so as to obtain agar plates containing 100-300 colonies. The plates were then subjected to ultraviolet irradiation at 254 nm for 30 seconds at a distance of 20 cm from the irradiation source (Vilbert Lourmat VL 30 LC) and then grown at 20-22°C for 10 days after which the colour of the resulting
20 colonies was compared.

EMS (Ethyl-Methane-Sulphonate) treatment

2x15 ml of a four days old culture of ATCC 24261 grown in YM medium on a shake board at a temperature of 20-22°C were centrifugated for 15 minutes at 1250 x g in a MSE Major centrifuge and the pellet was
25 suspended in 2x15 ml of sterile 0.9% NaCl solution in 200 ml centrifugation tubes. One of the cell suspensions was employed as a control. To the other cell suspension 1 g of EMS (Serva 28755) was added. After treatment for 30 minutes at 20°C, 150 ml of cold sterile

0.9% NaCl solution was added. The yeast cells were washed twice in sterile 0.9% NaCl and suspended in 0.9% NaCl. The yeast cell suspension was then diluted and plated on agar plates in the same manner as described above for the UV-treatment. One of the isolated mutants was deposited at the CBS (Centraalbureau voor Schimmelcultures) on 6 April, 1987 under the accession No. 224-87.

Treatment with N-methyl-N'-nitro-N-nitrosoguanidine

About 25 mg of N-methyl-N'-nitro-N-nitrosoguanidine (Aldrich Chemie BDR) were added to a 10 ml tared graduated cylinder supplied with a glass stopper. Water was supplied so as to obtain a total volume of 10 ml, and the N-methyl-N'-nitro-N-nitrosoguanidine was dissolved therein by shaking. 10x1 ml of stock solution were obtained from this solution.

2x20 ml of a four days old culture of CBS 224-87 (the mutant obtained by the above EMS treatment) grown in YM medium on a shake board at 20-22°C were transferred to Sarstedt tubes and subjected to two rounds of centrifugation. The pellet was suspended in 1.5 ml of 0.9% sterile NaCl solution and 1 ml of the stock solution prepared above was added. After incubation for 1 hour at 20-22°C, the yeast cells were washed 5 times in 10 ml of cold sterile 0.9% NaCl solution, whereby the N-methyl-N'-nitro-N-nitrosoguanidine was removed. The yeast cells were then diluted and plated on agar plates in the same manner as described above for the UV-treatment. One of the isolated mutants was deposited at the CBS on 6 April, 1987 under the accession No. 225-87.

Reisolation

CBS 225-87 has been subjected to isolation as described as follows. From a freeze-dried vial yeast cells are suspended in YM medium and incubated for 5 day at 20-22°C. The culture is plated on YM plates and incubated for 10 days at 20-22°C and new colonies are isolated. One of the colonies is deposited at the CBS on 23 March, 1988 under the accession No. 215-88.

EXAMPLE 2

Determination of the astaxanthin content of wild-type Phaffia rhodomyza strains and of mutants prepared in Example 1

The yeast cell cultivation and the astaxanthin determination described in the present Example constitute, on the one hand, the conditions under which the yeast cells are grown and on the other hand the conditions under which astaxanthin is determined in the applicant's above-mentioned standard method for determining the inherent astaxanthin-producing capability of a yeast strain. These are the same standard conditions as are referred to in the claims.

Shake flask cultivation

100 μ l of a 4 days old culture of ATCC 24261 grown in YM medium on a shake board at 20-22°C were inoculated in 50 ml of YM medium contained in a 500 ml shake flask with 2 baffles. The culture was subjected to growth on a shake board with orbital shaking at 150 rpm for 5 days at 20-22°C and at an oxygen transfer rate of 30 mmoles/l/hour, whereby a density of the yeast cell culture of 0.6% was obtained.

Pigment analysis

The astaxanthin content in the extract was identified by the following three methods.

1. An acetone, methanol and ethanol extract which had all been prepared as described for the methanol extract preparation above was subjected to spectrophotometric scanning and the λ_{\max} values stated in Table 1 were obtained.
- 25 2. To one half of a 10 ml ethanol extract, prepared in the same manner as above, about 50 mg of potassium borohydride were added to reduce the astaxanthin, and the mixture was stirred for 30 minutes. The absorptions of the extract and of the potassium borohydride-treated sample were measured with varying wavelengths on the spectro-

photometer. The free astaxanthin showed a broad peak at 480 nm and the reduced astaxanthin showed two peaks at 450 and 476 nm, corresponding to the values stated in the literature.

3. The retention time of the astaxanthin-containing extracts in HPLC under standard conditions as defined above was compared with the retention time of the standard solution defined above, i.e. the peaks of the astaxanthin-containing sample of the invention in HPLC under standard conditions were compared with the peaks of the standard solution in HPLC. The retention times were found to be identical.
- 10 The mutant strains of the invention (CBS 224-87 and CBS 225-87) as well as all known deposited astaxanthin-producing *P. rhododyma* strains were grown and analyzed in the same manner as described above. The total pigment content and the astaxanthin content of the strains are stated in Table 2 below.
- 15 Total pigment analysis were carried out according to method: pigment extraction and analysis - Method 1. Astaxanthin were analysed according to HPLC analysis for astaxanthin determination - Method 1.

TABLE 2

	Strain	μg of total pigment/g of yeast dry matter	μg of astaxan- thin/g of of yeast dry matter
5			
	CBS 5905 = ATCC 24202 = UCD 67-210	332	254
	CBS 5908 = ATCC 24203 = UCD 67-383	318	252
	CBS 6938	303	204
10	CBS 6954	< 50	<100
	ATCC 24201 = UCD 67-203	229	143
	ATCC 24203 = UCD 67-383	338	164
	ATCC 24228 = UCD 68-653C	254	107
	ATCC 24229 = UCD 67-202	287	142
15	ATCC 24230 = UCD 67-385	247	132
	ATCC 24261 = UCD 67-484	449	286
	CBS 224-87	885	570
	CBS 225-87	1176	706

- 20 The values are the means of 4 independent measurements. It will be noted that the mutant strains of the invention show a considerably increased astaxanthin content.

EXAMPLE 3

Fermentation

- 25 The fermentations were performed as fed-batch fermentations under carbohydrate limitation in thoroughly washed and sterilized 4 m³ fermenters of the type Bubble Column with a stationary aeration system consisting of perforated air pipes. The fermenters were equipped with pH electrodes, inlets for pH regulating agents and
- 30 foam-suppressing agents, and alcohol detectors for measuring alcohol in the discharged air. The jackets of the fermenters were thermostated.

The start wort has the following composition: 20 g/l of molasses, 0.6 g/l of diammonium sulphate, 0.8 g/l of diammonium hydrogenphosphate and 0.125 g/l of magnesium sulphate which altogether were boiled up in the fermenter for 30 minutes together with a suitable amount of water (30 l in the 100 l propagation fermenter and 2000 l in the 4 m³ production fermenter) before the fermenter in question was inoculated. The medium which was fed to the fermenter in the fed-batch fermentation was taken from two different reservoirs, i.e. a chemical reservoir consisting of 10 kg of diammonium sulphate, 5.6 kg of diammonium hydrogenphosphate and 80 l of water; and a molasses reservoir consisting of 450 kg of molasses and 1000 l of water which had been autoclaved. 0.1 mg of desthiobiotin and 1.6 kg of magnesium sulphate were supplied directly to the fermenter before the rest of the medium was supplied. All the chemicals were of food grade. The molasses were beet molasses from De Danske Sukkerfabrikker.

The aeration during the fermentation was 8.4 m³/minute. Contraspum 210 (Zschimmer & Schwartz) was employed as the foam-suppressing agent, and sulfuric acid was employed as the pH regulating agent.

Yeast cells of strain ATCC 24261 were propagated by being transferred from a slant to a test tube with a diameter of 2 cm containing 5 ml of YM medium in which the cells were cultured for 4 days on a shake board under sufficient aeration at a temperature of 20-22°C, after which the culture was transferred to 2 l Erlenmeyer flasks containing 1 l of YM medium. After incubation for 3 days on a shake board and under sufficient aeration at a temperature of 20-22°C, 1 l of the culture was transferred to a 100 l fermenter containing 30 l of start wort. The culture was subjected to batch growth at 20-22°C until a yeast dry matter content of 1 g/l was obtained. Thereafter, the nutrient supply was started and the fed-batch fermentation was performed at 20-22°C. After 2 days' growth, 30 l of the culture were transferred under sterile conditions by means of a peristaltic pump to the 4 m³ fermenter which contained 2000 l of start wort. The culture was subjected to batch growth at 20-22°C until a yeast dry matter content of 1 g/l in the culture was obtained. Then, the molasses supply was started and continued for 38 hours after which the molasses reservoir

was depleted. The chemicals were supplied proportionally with the molasses during the first 24 hours. The fed-batch fermentation was performed at a temperature of 20-22°C. The amount of molasses in the molasses reservoir was adjusted so that a yeast dry matter content of
5 not more than about 4% in the fermented wort would be obtained.

The molasses supply to the fermenter during the fed-batch fermentation was adjusted with the aim of reaching a specific growth rate of the yeast cells of $\mu = 0.15 \text{ hour}^{-1}$ and was further regulated in accordance with the ethanol concentration in the wort which should be
10 lower than 0.1% by volume. Thus, the ethanol concentration was frequently measured, and when it was found to be too high, the molasses supply rate was lowered until an acceptable ethanol content was again obtained.

The aeration of the fermented wort was continued for 16 hours at
15 20-22°C without any nutrient supply.

The composition of the yeast cells as well as the total pigment content and the astaxanthin content were measured at time 0, i.e. just before the nutrient supply to the 4 m³ fermenter was started, after 38 hours when the fermentation and growth had terminated, and after
20 16 hours' aeration of the fermented wort. The total pigment content and the astaxanthin content were determined as described in Example 2, and the composition of the yeast cells was determined by conventional techniques. Thus, the total content of nitrogen was determined by Kjeldahl analysis, the trehalose content was determined as described in *Journ. Am. Chem. Soc.* 72, 1950, p. 2059, and the phosphoric acid content was determined as described in *Water and Wastewater*, American Public Health Association, Inc., p. 199 (1960). Ethanol analysis was performed by Boidin's method for the determination of small amounts of alcohol (cf. *Annal. de la brasserie et de la*
25 *distillerie*, 1924-25, p. 177). The results are stated in Table 3 below.
30

Total pigment analysis were carried out according to method 1.

Astaxanthin analysis by HPLC were carried out according to method 1.

TABLE 3

Fed-batch fermentation of ATCC 24261

Hours	0	18	24	31	38	45	54
5 μg of total pigment/g of yeast dry matter	-	181	218	284	415	471	579
μg of astaxanthin/g of yeast dry matter	-	110	-	-	230	300	350
% w/w yeast dry matter	0.08	0.48	0.95	2.75	3.06	3.15	3.29
10 % v/v ethanol	-	0.00	0.00	0.00	0.10	0.00	0.00
pH	4.7	4.4	4.0	4.0	4.5	7.8	8.5
% w/w N in yeast dry matter	-	-	-	-	6.2	6.1	5.7
15 % w/w P_2O_5 in yeast dry matter	-	-	-	-	2.7	2.6	2.4
% w/w trehalose in yeast dry matter	-	-	-	-	2.6	4.4	11.4

EXAMPLE 4

20 In a manner similar to the experiment described in Example 3, fed-batch fermentations with strain ATCC 24261 were carried out, the only differences being that the start wort volume was 1000 l, the inoculum in the 4 m³ fermenter was 6 x 1 l of ATCC 24261 which had been propagated as stated above, and the chemical reservoir consisted

25 of 0.5 kg of diammonium sulphate, 2.8 kg of diammonium hydrogenphosphate and 80 l of water, and the molasses reservoir consisted of 250 kg of molasses and 1000 l of water. During the fed-batch fermentation, the nutrient was fed to the fermenter for the first 65 hours, after which the nutrient supply was terminated and

30 the culture was subjected to aeration for 72 hours.

The results are stated in Table 4 below.

TABLE 4

Fed-batch fermentation of ATCC 24261

Hours		0	65	137
5	μg of total pigment/g of yeast dry matter	149	379	561
	μg of astaxanthin/g of yeast dry matter	89	231	369
	% w/w yeast dry matter	0.05	3.5	3.75
10	% v/v ethanol	-	0.0	-
	pH	4.5	7.8	9.1
	% w/w N in yeast dry matter	-	4.1	4.5
	% w/w P_2O_5 in yeast dry matter	-	1.9	2.2
15	% w/w trehalose in yeast dry matter	2.6	13.2	12.8

EXAMPLE 5

Experiments with the mutant strain CBS 225-87 of the invention were carried out in the same manner as described in Example 4 with a start wort volume of 1000 l, the inoculum being 6 x 1 l of CBS 225-87 which had been propagated by the method described in Example 3 and with a chemical reservoir consisting of 5 kg of diammonium sulphate, 2.8 kg of diammonium hydrogenphosphate and 80 l of water. No alcohol was formed during the fed-batch fermentation, and the nutrient was supplied between hours 0-57 after which the culture was subjected to aeration without nutrient supply. The yeast cell composition at hour 80 was 6.5% nitrogen in yeast dry matter, 2.5% phosphorous pentoxide in yeast dry matter and 6.6% trehalose in yeast dry matter. The total pigment, the astaxanthin and the yeast dry matter were determined

during the fed-batch fermentation, giving the values stated in Table 5 below:

TABLE 5

Fed-batch fermentation of CBS 225-87

5	Hours	Yeast dry	Total pigment		Astaxanthin	
		matter	in	in	in	in
			sample	yeast	sample	yeast
		g/l	$\mu\text{g/ml}$	$\mu\text{g/g}$	$\mu\text{g/ml}$	$\mu\text{g/g}$
10	0	1.4	0.9	640		
	22	11.2	8.1	720		
	33	18.4	12.5	680		
	37	20.0	15.6	780	11.0	550
	40	20.8	16.5	790	8.8	420
15	57	23.8	24.0	1010	16.2	680
	61	24.7	24.1	980	17.0	690
	64	24.3	25.5	1050	17.3	710
	80	24.6	36.6	1490	23.6	960

20 The total pigment and astaxanthin content in $\mu\text{g/ml}$ has been calculated from the analyzed yeast dry matter content and $\mu\text{g/g}$ -values of total pigment and astaxanthin.

EXAMPLE 6

25 The pigment and astaxanthin content of CBS 215-88 and *P. rhodozyma* mutant strains DBT 406 and DBT 403, the wildtype strains CBS 5905 and ATCC 24261 were determined.

Shake flask cultivations

30 From an agar slope, yeast cells were inoculated in YM-medium and incubated for 2 days at 20-22°C. 1 ml of the culture was inoculated in 50 ml of YM-medium, contained in 250 ml shake flasks with 4

baffles. The culture was subjected to growth on a shake board with orbital shaking at 150 rpm for 5 days at 20-22°C.

Quantitative determinations of total pigment were carried out according to method 2. Astaxanthin determination were carried out
5 according to method 2.

Example of calculations (for CBS 215-88):

The total pigment in the methanol extract was determined by spectrophotometrical analysis. The absorption of the 50 ml extract minus absorption of methanol was measured to be $E = 1.189$. The volume
10 of the sample was 29 ml. The total pigment content in the yeast extract calculated by formula (2) as follows:

$$X' = 1.160 \times 50 / 2100 / 29 \times 10.000 \mu\text{g/ml} = 9.52 \mu\text{g/ml}$$

The yeast dry matter was determined to be 6.7 g/l. The total pigment content per g of yeast dry matter is determined by formula (2a) as
15 follows:

$$Y = 9.52 / 7.1 \times 1.000 \mu\text{g/g} = 1340 \mu\text{g/g}$$

the concentration of astaxanthin in the methanol was determined by HPLC analysis to be 6.4 $\mu\text{g/ml}$ corresponding:

$$6.2 \mu\text{g/ml} / 7.1 \text{ g/l} = 880 \mu\text{g astaxanthin} / \text{g yeast dry matter}$$

20 All strains were grown and analysed in the same manner as described above. The total pigment and astaxanthin content of the strain are stated in Table 6.

TABLE 6

	Strain	Yeast dry matter g/l	Total pigment		Astaxanthin	
			$\mu\text{g/ml}$	$\mu\text{g/g}$	$\mu\text{g/ml}$	$\mu\text{g/g}$
5	CBS 5905	5.3	1.38	260	0.84	160
	ATCC 24261	5.5	2.10	380	1.38	250
	DBT 406	2.2	5.84	2650	3.4	1540
	DBT 403	1.6	5.04	3150	3.3	2050
10	CBS 215-88	7.1	9.52	1340	6.2	880

EXAMPLE 7

Fed-batch fermentation of the mutant strain CBS 215-88 of the invention were carried out using corn steep solid and sucrose as carbohydrate sources in the same 4 m³ fermenter as described in Example 3. The start wort consisting of

	10	kg	corn steep solid
	12	-	sucrose
	10	-	diammonium sulphate
	3	-	potassium dihydrogen phosphate
20	1	-	magnesium sulphate
	0.05	g	biotin
	300	ml	antifoam Contraspum 210
	1000	l	water

was sterilized at pH 4.6 by injection of steam at 95°C for one hour and after cooling to 22°C 6x1 l CBS 215-88 propagated as stated above was added as inoculum. After 35 hours' of aeration (4.2 m³/minute) supply of sucrose solution (0.30 g/l) was started. The addition rate was 2.3 l/hour. Aeration was increased to 8.4 m³/minute and the pH-controller started (set point 4.0). When the sucrose concentration in the medium was decreased to about 1 g/l after 28 hours, the sucrose supply was increased to 7.3 l/hour and kept at this rate for 24 hours. Thereafter sucrose supply was terminated and the aeration rate was decreased to 4.2 m³/minute and continued for 72 hours. The total pigment and astaxanthin were determined during the fed-batch fermentation, giving the values stated in Table 7.

TABLE 7

Fed-batch fermentation of CBS 215-88

Hours of sucrose supply		28	52	
5	Hours of aeration after termination of sucrose supply			6
	μg of total pigment/ml culture medium	14.1	37.7	43.4
10	μg of astaxanthin/ml culture medium	-	23.4	29.9
	pH	4.0	4.0	4.0
	% w/w N in yeast dry matter	-	-	7.69
	% w/w P_2O_5 in yeast dry matter	-	-	3.72
15	% w/w trehalose in yeast dry matter	-	-	4.4

The yeast cells were separated from the medium by means of centrifugation in a De Laval OA5M centrifuge and washed with water twice. The yeast was separated from the yeast cream by means of filtration in a FILTROX-filter, type VARIOX 40/40 cm and the filter cake with 26.3% dry matter was extruded through a 1 mm sieve in a lab fluid bed dryer (GLATT, Haltingen-Binzen Bd.) and dried at 30°C for 90 minutes. The dried yeast (91.6% of dry matter) contained

1360 μg total pigment/g yeast dry matter

25 and

1080 μg astaxanthin/ yeast dry matter

EXAMPLE 8

Downstream processing

Yeast cells obtained by the method of Example 3 were isolated from the fermented wort by centrifugation in a De Laval OA5M centrifuge.

5 The cells were washed twice with water and a yeast cream with 13% of yeast dry matter was obtained. The pH of the yeast cream was adjusted to 4.0 by addition of sulphuric acid, and sodium benzoate was added to a concentration of 0.2% (w/v) in the yeast cream. During the treatment of the isolated yeast cells, these were under a nitrogen

10 cover so as to prevent substantial oxidation of the astaxanthin of the yeast cells, and the temperature was kept at about 10°C.

The yeast cream was subjected to three passages through a system consisting of an APV-Gaulin MC4 homogenizer provided with a cell rupture valve and a heat exchanger where the yeast cream was

15 subjected to disintegration at a pressure of 700 bar, whereby the temperature increased by 10-15°C and subsequent cooling in the heat exchanger so as to obtain a temperature of 15°C. The yeast cream was circulated in the system at a rate of 250 l/h.

The astaxanthin content in the disintegrated cells was determined as follows: 0.5 ml of homogenized yeast cream was weighed out and transferred to a Sarstedt tube and shaken with 5 ml of acetone. The sample was centrifugated, transferred to a 10 ml graduated cylinder, and washed 3 times with acetone with intervening centrifugations and quantitative transfers. The total pigment content was determined by

20 method 1 and astaxanthin content was determined by HPLC-method 1, and the content was related to the total dry matter content in the sample which was determined by the method explained in Materials and Methods above. By comparing the extractable astaxanthin content in the yeast cream homogenized according to the present Example with the

25 content in yeast cream determined by the method 1 where the cells were completely disintegrated, the degree of disintegration in the

30

present Example was determined to be more than 90% of the total cells.

To the cells thus disintegrated, which cells were covered with nitrogen, 7% of sodium caseinate was added at a temperature of about 45°C while stirring. The yeast cell homogenate in admixture with sodium caseinate was then subjected to spray drying in a spray tower of the type Anhydro in which the inlet temperature was 180°C. The yeast cell mass was atomized by use of a spray wheel and the temperature of the air let out of the spraytower was of a temperature of about 90°C. The resulting yeast powder was recovered by use of a cyclone. The water content in the yeast powder was less than 10% by weight.

EXAMPLE 9

Extraction of total pigment with glacial acetic acid

20 g of non-ruptured *Phaffia rhodozyma* yeast cells (which had been filtered and subsequently extruded into a fluid bed wherein they had been dried) having a dry matter content of 95% and containing 523 µg astaxanthin/g of yeast dry matter were introduced into a column of a length of 12 cm and an inner diameter of 2.4 cm. The column was equipped with a jacket wherein water of a temperature of 75°C was circulated. At the bottom of the column, a small amount of acid-washed sand (a sand filter) was arranged on a cotton layer. The yeast cells were extracted with 5 x 100 ml of glacial acetic acid at a temperature of 75°C, and the amount of astaxanthin in each of the extracts as well as in the extracted yeast cell material (including about 100 ml of glacial acetic acid remaining in the column) was determined. The extracted yeast cell material had been evaporated to dryness (resulting in 16.16 g of material) before the astaxanthin determination was carried out. The results are stated in Table 8 below.

TABLE 8

<hr/>		
Total astaxanthin content in yeast cell		
5	prior to extraction	20 g x 523 $\mu\text{g/g}$ 10460 μg
<hr/>		
Astaxanthin content in		
	1. extract	100 ml x 55.7 $\mu\text{g/ml}$ 5570 μg
	2. extract	100 ml x 14.6 $\mu\text{g/ml}$ 1460 μg
10	3. extract	100 ml x 4.4 $\mu\text{g/ml}$ 440 μg
	4. extract	100 ml x 3.1 $\mu\text{g/ml}$ 310 μg
		<hr/>
Total astaxanthin content of extracts		7780 μg
15	Astaxanthin content of extracted yeast cell material	
	16.16 g x 22.5 $\mu\text{g/g}$	363.6 μg
		<hr/>
20	Total astaxanthin content released from yeast cells by the extraction	
		8143.6 μg
<hr/>		

25 Thus, 77.9% ($8143.6/10460 \times 100\%$) of the astaxanthin content of the
yeast cells was released by the extraction.

EXAMPLE 10

Rupturing of yeast cells by homogenization in a ball mill

30 *Phaffia rhodozyma* yeast cells, which had been dried in a fluid bed
and which was found to contain 336 μg of astaxanthin/g of yeast dry
matter, was suspended in soy bean oil in a concentration of 40%

(w/w). The suspension was pumped to a ball mill (CoBall® - Mill, type MSZ-12) containing zirkonium balls (0.1-1.5 mm) and having a bead-filling of 70-75%. The bead of the rotor was 13 m/sec. Samples were taken after each run, and the astaxanthin content of the samples was analyzed on HPLC. The temperature in the ball mill was kept at 40-50°C.

Similarly, a suspension of the above dried yeast cells in 75% water was treated in the ball mill. In this treatment, the speed of the rotor was 15 m/sec.

10 The results are stated in Table 8, wherein the astaxanthin content is stated as $\mu\text{g/g}$ of yeast dry matter.

TABLE 9

	60% soy bean oil	75% water
15 1st run	221	179
2nd run	308	192
3rd run	313	276

20 For comparison, only 23 μg of astaxanthin/g of yeast dry matter was found when the dried yeast was treated with soy bean oil or water without the simultaneous homogenization.

By the experiment it is shown that about 3 runs in the ball mill are sufficient to obtain a substantially total rupture of the yeast cell.

EXAMPLE 11

25 *Feeding of fish*

- Fish feed of varying astaxanthin contents were prepared. The fish feed was made from the commercial fish feed Ecoline 16 from Dansk Ørredfoder, Brande, Denmark, which is a mixture of fish meal, soy meal, fish oil, extruded wheat, lecitin and vitamins in the form of a premix. Various amounts of astaxanthin were added to this feed. The astaxanthin was obtained from *P. rhodozyma* yeast cells which had been grown in the same manner as described in Example 3 and which had been spray dried. The spray dried yeast cells were prepared from 28 kg homogenized yeast cream which had been mixed with 0.475 kg of sodium caseinate dissolved in 2.7 kg water at about 50°C. 0.068 kg GRINDTEK MOR 50 containing 2 g of ascorbyl palmitate and 1 g of tocopherols from soy beans was emulgated in the sodium caseinate solution. The sodium caseinate solution (containing antioxidants) was mixed with the homogenized yeast cream and the mixture was spray dried as described in Example 8. The spray dried product (92.6% dry matter) contained about 674 µg of astaxanthin/g of yeast dry matter. The spray dried product was added to the commercial fish feed so as to obtain the varying concentrations of astaxanthin in the fish feed (feed A-D) which appear from table 10 below.
- 20 A fish feed containing synthetic astaxanthin (feed E) was employed as a control. The feed E contained synthetic astaxanthin in an amount corresponding to 40 ppm.

The feeds A-E had the following composition:

TABLE 10

Fish feed

	A	B	C	D	E
5 Astaxanthin $\mu\text{g/kg}$ feed	4.4	12.8	20.4	39.2	-
Synthetic astaxanthin	-	-	-	-	40
Dry matter %	91.94	91.49	91.56	91.88	88.81
Ash %	9.12	9.00	8.79	8.45	7.03
10 cellulose %	1.46	1.45	1.60	2.05	1.71
Protein %	43.62	43.20	43.86	43.81	43.15
Fat %	17.59	17.21	18.62	20.00	21.19
Phosphor g/kg	11.58	10.89	11.25	10.80	9.96
15 Nitrogen-free extract %	20.15	20.53	18.69	17.57	15.73

About 60 rainbow trouts, each of a weight of about 400 g, were used in the experiments with each of the fish feed A-E. The fish were kept in cages and fed ad libitum. The water was of a temperature in the range of 2.5-14°C, the lower temperatures in the last part of the fish feeding experiment.

Extraction of astaxanthin from fish

The equipment used is equipment conventionally used in laboratory experiments.

A rainbow trout without skin was cut into pieces and 15 g of flesh were weighed out in a centrifugal tube (100 ml). 15 ml of tetrahydrofurane were added as extraction agent. The flesh was further divided in an ULTRATURAX mixer and subsequently centrifugated. The tetrahydrofurane extract was transferred to a 50 ml measuring flask. The remanence was washed with 10 ml of tetrahydrofurane for 2-3 minutes on a sonication bath and centrifugated, and the tetrahydrofurane phase was transferred to the measuring flask to which additional tetrahydrofurane was added up to

50 ml. 10 ml out of the 50 ml were subjected to evaporation under a nitrogen cover at a temperature of 40°C. The evaporation residue was redissolved in 1 ml of mobile phase and filtered through a 0.45 µm filter prior to further analysis.

5 HPLC analysis

Column:	LiChrosorb RP-18, 5 µm, 250 x 4.6 mm
Mobile phase:	40 ml formic acid
	60 ml water
	384 ml ethylacetate
10	516 ml acetonitrile
Flow:	10 ml/min.
Injection:	20 µl Loop, Rheodyne 7120
Pump:	Waters 510
Detector:	Waters 481, UV-spectrophotometer, 471 nm
15	Integrator: Waters 740

Determination of the colour of the fish

The colour of the fish flesh was determined by the L*a*b*-colour determination method by use of a Minolta Chroma Meter II. The L*-value designates the light component, the a*-value (ranging from -60 to +60) designates the green/red component (the negative values designating the green component and the positive values designating the red component), and the b*-value (ranging from -60 to +60) designates the blue/yellow component of the colour. Only the a*-value is stated in Table 11.

25 Rainbow trout flesh without skin was homogenized in a blender to obtain a homogeneous mass which was put into a small petri-dish (of a height of 1 cm and a diameter of 3.5 cm) so as to occupy the total volume of this. The surface of the mass in the petri-dish was smoothened out and covered with a glass plate, and was then ready for
30 analysis.

In the following Table 11, the data of the fish feeding experiment are stated:

TABLE 11
Analysis of fish fed for 16 days

		μg of asta- xanthin/g of fish	weight of fish in g	μg of fish flesh (a*)
5				
	Feed A	0.50	380	1.45
		0.60	434	1.12
		0.65	252	0.08
10		0.60	580	0.48
		0.65	392	-0.02
	Feed B	1.45	343	1.53
		0.15	385	0.05
		0.20	298	-0.52
15		1.00	388	0.52
		0.60	317	-0.33
	Feed C	0.75	471	0.00
		0.85	290	1.20
		0.45	328	0.23
20		0.30	300	-0.38
		0.75	359	-0.38
	Feed D	0.70	258	-0.20
		0.45	643	0.72
		1.00	369	2.05
25		0.70	450	0.50
		0.30	382	-0.10
	Feed E	0.25	374	-0.80
		0.60	339	0.12
		0.80	405	0.98
30		0.65	579	0.36
		1.20	534	1.18

TABLE 11 - continued
Analysis of fish fed for 23 days

5		μg of asta- xanthin/g of fish	weight of fish in g	μg of fish flesh (a*)
10	Feed A	0.65	668	0.76
		0.60	571	1.00
		0.40	535	0.26
		0.50	469	-0.50
		0.20	386	0.06
15	Feed B	0.40	348	0.02
		0.40	325	0.16
		0.75	500	1.32
		0.75	355	0.16
		0.50	406	0.18
20	Feed C	1.25	356	1.25
		1.05	464	1.88
		1.05	317	1.98
		0.25	301	-1.44
		0.50	329	-0.44
25	Feed D	0.50	287	-0.14
		0.65	380	1.34
		0.45	436	0.62
		1.65	449	3.38
		1.85	409	3.26
30	Feed E	2.00	487	4.08
		0.50	429	0.82
		0.35	673	2.53
		1.20	441	3.16
		1.15	404	0.90

TABLE 11 - continued
Analysis of fish fed for 30 days

		μg of asta- xanthin/g of fish	weight of fish in g	μg of fish flesh (a*)
5				
	Feed A	0.80	410	1.12
		0.60	448	0.78
		0.50	409	1.50
10		0.55	483	1.40
		0.65	352	0.40
	Feed B	0.75	344	0.35
		0.35	410	1.17
		0.45	547	0.40
15		0.35	493	2.20
		0.95	228	2.14
	Feed C	1.10	517	3.30
		0.95	405	1.48
		1.55	381	2.26
20		0.75	330	1.48
		0.95	413	1.80
	Feed D	2.15	635	6.10
		0.85	384	1.68
		2.10	363	0.56
25		1.70	423	3.70
		1.00	348	1.92
	Feed E	1.15	390	1.84
		2.10	427	4.00
		3.00	433	4.14
30		0.40	337	0.02
		0.55	342	0.85

TABLE 11 - continued
Analysis of fish fed for 43 days

5		μg of asta- xanthin/g of fish	weight of fish in g	μg of fish flesh (a*)
10	Feed A	1.00	429	2.50
		0.60	300	-0.56
		0.90	848	2.32
		0.50	417	0.16
		0.45	385	-0.54
15	Feed B	0.75	623	1.94
		1.00	352	1.88
		1.00	620	1.44
		0.75	484	1.52
		0.95	441	0.78
20	Feed C	0.75	604	1.30
		0.90	480	1.84
		1.20	540	2.96
		2.20	444	4.78
		1.35	414	0.74
25	Feed D	1.45	471	3.66
		2.10	436	6.61
		2.15	508	5.56
		3.00	510	6.04
		1.15	381	1.72
30	Feed E	1.20	512	2.40
		3.00	452	4.84
		4.30	634	8.04
		1.75	474	4.43
		3.70	517	5.46

TABLE 11 - continued
Analysis of fish fed for 72 days

	μg of asta- xanthin/g of fish	weight of fish in g	μg of fish flesh (a*)	
5				
	Feed A	0.55	409	1.08
		0.70	628	4.02
		1.95	1177	3.84
10		0.70	663	1.42
		1.65	401	1.34
	Feed B	1.05	386	2.24
		0.90	666	2.46
		0.30	507	2.46
15		2.05	585	4.90
		1.20	518	1.52
	Feed C	1.15	701	2.40
		1.80	415	4.42
		4.80	739	9.36
20		5.00	451	7.26
		4.00	594	5.48
	Feed D	1.15	444	2.32
		2.05	514	6.98
		4.25	493	8.40
25		4.80	612	8.24
		4.25	633	6.66
	Feed E	3.05	627	6.32
		3.75	618	6.26
		4.65	507	9.78
30		5.95	654	8.56

The results show that the astaxanthin of each of the fish feed A-E has been absorbed by the fish and that the fish flesh obtains an increasing red pigmentation with increasing amounts of astaxanthin in the feed (feed A-D) and with increasing time of feeding (as observed by the a^* -value (designating the red component of the colour)).

Further, the above results indicate that the presence of astaxanthin in the feed do not affect the growth of the fish.

The fish were also subjected to visual examination and were generally found to be of an attractive red colour. After 43 days of feeding, substantially no difference was observed in the pigmentation of fish fed with feed D and E (containing about 40 ppm astaxanthin produced according to the present invention and 40 ppm synthetic astaxanthin, respectively). After 72 days of feeding, substantially no difference was observed in the pigmentation of fish fed with feed C, D and E (containing about 20 ppm astaxanthin produced according to the present invention, 40 ppm astaxanthin produced according to the present invention, and 40 ppm synthetic astaxanthin, respectively).

EXAMPLE 12

Fish paté

- 15 A fish paté (salmon-like) in which astaxanthin is used to impart the red colour can be made according to the following recipe:

	Cod scraps	71%
	Oil	3%
	Rusk	4%
20	Grindsted Protein 177*)	1%
	Starch	1%
	Astaxanthin	0.001%
	Water, preservatives and spices up to	100%

- 25 *) Grindsted Protein 177 is a blend of 75% of Grindsted Protein 100 and 25% of sodium alginate.

The astaxanthin was dispersed in the oil phase. The Grindsted Protein 177, starch and other dry ingredients were mixed, and the fish were added to a colloid mill. Then, the oil phase, the dry ingredients and water were added, and processing was continued in the colloid mill

58

for about 10 minutes. The paté was filled into tins and subjected to heat treatment.

Red dressing

A red dressing with an attractive red colour can be prepared by
5 conventional methods from the following ingredients:

	Oil	30.0%
	Tarragon vinegar	12.3%
	Tomato paste	8.0%
	Mayodan DC *)	0.3%
10	Sugar	8.0%
	Salt	0.8%
	Astaxanthin	0.01-0.5%
	Water, preservatives and spices up to	100%

15 *) Mayodan DC® is a stabilizer blend from Grindsted Products A/S.

CLAIMS

1. A yeast cell which, when grown under conditions comprising an oxygen transfer rate of at least 30 mmol/l/hour on Difco YM medium at 20-22°C for 5 days in 500 ml shake flasks with two baffles containing
5 50 ml of the medium and subjected to orbital shaking at 150 rpm, the inoculum being 100 μ l of a four days old YM culture, produces astaxanthin in an amount of at least 300 μ g per g of yeast dry matter, determined by HPLC analysis using pure astaxanthin as a standard on a
10 0.2 g of yeast dry matter in 20 ml of methanol to 5 x 1 minutes of disintegration at intervals of half a minute, the disintegration being performed at a temperature of at the most 20°C in a glass ball mill containing 15 g of glass balls having a diameter of 0.4 mm, the glass ball mill being provided with a cooling jacket with ice water.
- 15 2. A yeast cell according to claim 1 which belongs to the genus *Phaffia*, and preferably to the species *Phaffia rhodozyma*.
3. A yeast cell according to claim 2 which is a yeast cell belonging to the yeast strain deposited under the accession No. 224-87 CBS, to the yeast strain deposited under the accession No. 225-87 CBS, or the
20 yeast strain deposited under the accession No. 215-88 CBS, or a mutant or derivative thereof which has retained its astaxanthin-producing capability.
4. A yeast cell according to any of the preceding claims which, when grown under the conditions stated in claim 1, produces astaxanthin in
25 an amount of at least 450 μ g per g of yeast dry matter, such as at least 600 μ g per g of yeast dry matter, preferably at least 700 μ g per g of yeast dry matter, more preferably at least 1000 μ g per g of yeast dry matter, especially at least 1500 μ g per g of yeast dry matter, and most preferably at least 2000 μ g per g of yeast dry
30 matter determined by the method stated in claim 1.

5. A method for producing a yeast cell according to any of claims 1-4, comprising treating a yeast cell with a mutagen and selecting a resulting mutant which, when grown under the conditions stated in claim 1, is capable of producing astaxanthin in an amount of at least 300 μg per g of yeast dry matter, determined by the method stated in claim 1.
6. A method according to claim 5, wherein the yeast cell is subjected to two or more consecutive mutagenizations.
7. A method as claimed in claim 5 or 6, wherein the yeast cell subjected to mutagenization is a yeast cell which, when grown under the conditions stated in claim 1, produces astaxanthin in an amount of less than 300 μg per g of yeast dry matter, determined by the method stated in claim 1.
8. A method according to any of claims 5-7, wherein the mutagen is selected from ethyl methane sulphonate, UV irradiation and N-methyl-N'-nitro-N-nitrosoguanidine.
9. A method for producing astaxanthin-containing yeast cells or cell parts or astaxanthin, comprising cultivating astaxanthin-producing yeast cells under aerobic conditions in a medium containing carbohydrate sources, assimilable sources of nitrogen and phosphorus, micronutrients and biotin or desthiobiotin at a temperature in the range of 15-26°C so as to obtain a biomass containing astaxanthin in an amount of at least 300 μg per g of yeast dry matter, determined by the method stated in claim 1, and optionally performing one or several of the following steps in arbitrary sequence:
- harvesting cells from the culture so as to obtain a yeast cream,
 - opening the cells, for example rupturing the cell walls by means of mechanical, chemical and/or enzymatic treatment and/or subjecting the cells to sonication, autolysis, osmolysis and/or plasmolysis optionally with addition of suitable agents such as detergents, acids, bases, enzymes, autolysis-enhancing substances, osmolysing agents such as salts, and/or plasmolysing agents,

- homogenizing the cells to obtain a homogenate,
- drying the cells, the cell fragments or the homogenate, preferably to a water content of at the most 12% by weight, preferably at the most 10% by weight,
- 5 - extracting astaxanthin from the cells, the cell fragments or the homogenate.

10. A method according to claim 9, wherein the cultivation is performed as a fed-batch fermentation under conditions where substantially no alcohol is formed, and optionally where the total
10 concentration of glucose and saccharose is at the most 8 g/l, preferably 5 g/l and most preferably 1 g/l, preferably at a temperature of 20-22°C, the fermentation or part thereof is performed in a medium which comprises molasses and/or saccharose and nitrogen
15 sources such as diammonium sulphate, ammonium phosphate, ammonium hydroxide or urea, phosphorus sources such as ammonium phosphate and phosphoric acid and added micronutrients or mineral salts such as magnesium sulphate, zinc sulphate and biotin or desthiobiotin, the molasses optionally being supplied to the medium separately from the other components.

20 11. A method according to claims 9 or 10, wherein the cultivation comprises a growth phase under conditions which are substantially sufficient with respect to substantially all growth conditions and a subsequent growth-limited phase under conditions where the growth medium under continued aeration is deprived of at least one growth
25 factor so as to enhance the production of astaxanthin during the subsequent phase.

12. A method according to claim 11, wherein the subsequent growth-limited phase has a duration of at least about 16 hours, such as 16-24 hours, the subsequent growth-limited phase preferably being
30 adapted to enhance the astaxanthin production to at least 1.2 times the production obtained without the subsequent phase, preferably to at least 1.3, such as 1.4, and more preferably 1.5 times the production obtained without the subsequent phase, the subsequent growth-limited phase preferably being performed substantially without
35 any addition of any nutrient or micronutrient to the medium.

13. A method according to any of claims 9-12, wherein the yeast cell subjected to cultivation is a wild-type astaxanthin-producing yeast cell, preferably a yeast cell belonging to the genus *Phaffia*, such as a yeast cell belonging to the species *Phaffia rhodozyma*.
- 5 14. A method according to any of claims 11-13, wherein the concentration of astaxanthin in the biomass obtained is at least 250 μg per g of yeast dry matter, preferably at least 300 μg per g of yeast dry matter, determined by the method stated in claim 1.
- 10 15. A method according to any of claims 5-12, wherein the yeast cell subjected to cultivation is a yeast cell as claimed in any of claims 1-4.
- 15 16. A method according to claim 15, wherein the concentration of astaxanthin in the biomass obtained is at least 600, preferably at least 800, more preferably at least 1000 μg per g of yeast dry matter, especially at least 1500 μg per g of yeast dry matter, e.g. at least 2000 μg per g of yeast dry matter, and most preferably at least 3000 μg per g of yeast dry matter, determined by the method stated in claim 1.
- 20 17. A method according to any of claims 9-16, wherein the yeast cells, subsequently to the cultivation and the optional subsequent growth-limited phase, are subjected to one or several of the following treatments:
- rupturing the cells by subjecting the cells to an increased pressure and then releasing the pressure, and subsequently subjecting
 - 25 the ruptured cells to ultrafiltration or evaporation so as to concentrate the ruptured yeast cells,
 - rupturing the cells by subjecting the cells to an increased pressure and then releasing the pressure, and subsequently spray drying or drum drying the ruptured cells,
 - 30 - filtration so as to obtain a filter cake which is then extruded, after which the extrudate is dried, for example in a fluid bed or by tray drying,

- mixing whole cell dried material which has been dried in a fluid bed or by tray drying with an oily phase such as edible oil or fat, and grinding the mixture in a mill such as a ball stirring mill to rupture the cells and release astaxanthin into the oily phase,
- 5 - extracting ruptured cells preferably homogenized with organic solvents such as petroleum ether, acetone, or an alcohol such as methanol, ethanol or isopropanol to obtain astaxanthin dissolved in the organic solvent, and then optionally removing the solvent such as by evaporation,
- 10 - extracting whole cells with a solvent comprising glacial acetic acid to obtain astaxanthin contained in the glacial acetic acid-containing solvent,
- supercritical extraction of ruptured or whole wet or dry cells with carbondioxid, optionally in combination with organic solvents, e.g of
- 15 the above mentioned type.

18. A method according to claim 17, wherein the treatments are performed under oxygen-limited conditions such as in an inert atmosphere, e.g. the inert atmosphere being established by means of water vapour and/or by means of nitrogen and/or carbon dioxide.

- 20 19. A method according to claim 17 or 18, wherein the extraction of the whole yeast cells and the glacial acetic acid-containing solvent is performed at a temperature above the freezing point of the solvent, e.g. in the range of 20-100°C, preferably in the range of 20-80°C and more preferably in the range of 20-60°C.

- 25 20. A method according to any of claims 17-19, wherein the whole cell material and/or the extracted astaxanthin is protected by means of antioxidants such as butyl hydroxyanisol (BHA), butyl hydroxytoluene (BHT), vitamin E, ascorbic acid, or (II) sulphate or (II) phosphate esters of ascorbic acid, and/or emulsifiers such as monoglycerides or
- 30 sorbitan esters.

21. An animal feed comprising yeast cells or yeast cell parts containing astaxanthin in an amount of at least 300 µg per g of yeast dry matter, determined by the method stated in claim 1, in combination with other feed constituents, preferably an animal feed,

wherein the astaxanthin-containing yeast cells or yeast cell parts constitute at the most 10% by weight of the total animal feed composition dry matter, preferably at the most 5%, and more preferably at the most 3%, the other components preferably being
5 selected from protein and carbohydrate sources such as fish meal, whey, blood meal, vegetable flours, fats such as fish oil and vegetable oils, vitamins and minerals.


22. An animal feed according to claim 21, wherein the astaxanthin-containing yeast cells or yeast cell parts are yeast cells according to any of claims 1-4 or parts thereof or yeast cells or yeast
10 cell parts produced by the method according to any of claims 5-20.

23. A food or feed comprising astaxanthin which has been extracted by the method according to any of claims 17-20 from astaxanthin-containing yeast cells produced by the method of any of claims 5-16 combined
15 with other nutrient components such as proteins, carbohydrates and/or fats, preferably the astaxanthin is contained in an oily phase produced by the method of claim 26.

24. A method for feeding animals to obtain a reddish pigmentation of their meat and/or of products produced by the animals, comprising administering to the animals a feed containing yeast cells or cell
20 parts containing astaxanthin in an amount of at least 300 μg per g of yeast dry matter, determined by the method stated in claim 1, or astaxanthin extracted from such yeast cells or cell parts, the animals preferably being fish, especially salmon or sea trout, cows to
25 pigment their butter, or poultry to pigment their egg yolks.

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK88/00068

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC ⁴		
C 12 N 1/16, C 12 P 23/00, A 23 K 1/18 // C 12 R 1:645		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System ¹	Classification Symbols	
IPC 4	C 12 N 1/16; C 12 P 23/00; A 23 K 1/18	
US C1	435:255	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *		
SE, NO, DK, FI classes as above. Database search: CA, Biosis, WPI/L, US claims		
III. DOCUMENTS CONSIDERED TO BE RELEVANT *		
Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	Aquaculture, 20,1980, Johnson Eric A. et al, "Phaffia Rhodozyma as an astaxanthin source in salmonid diets" (see page 124, line 17, page 129, line 15-19)	1-24
X	Journal of General Microbiology, 1979,115, Johnson Eric A et al, "Astaxanthin Formation by the Yeast Phaffia rhodozyma" page 173-183 (see page 175, line 24, page 181 line 23-24 and table 1)	1-24
X	Applied and environmental Microbiology, Vol 35, No 6, June 1978, Johnson Eric A et al "Simple method for the isolation of astaxanthin from the basidiomycetous Yeast Phaffia rhodozyma" page 1155-1159, (see page 1157, right column line 7)	1-24
X	Journal of Applied Bacteriology, 1985, 59, Okagbue R.N. et al "Influence of mixed culture conditions on yeast-wall hydrolytic activity of Bacillus circulans WL-12 and on .../...	1-24
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
1988-06-15	1988 -07- 0 1	
International Searching Authority	Signature of Authorized Officer	
Swedish Patent Office	 Yvonne Siösteen	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	extractability of astaxanthin from the yeast Phaffia rhodozyma" page 243-255	
X	Patent abstracts of Japan vol 7, no 56 (C-155) 1201, Dec. 17, 1982, JP A 57-206342 (Sanraku Ocean KK)	21-24